CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

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2/20/06 David L. Parker
Date

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gong et al.

Serial No.: 10/605,708

Filed: October 21, 2003

For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

Group Art Unit: 1632

Examiner: Singh, Anoop Kumar

Atty. Dkt. No.: GLOF:007USC1

DECLARATION OF ZHIYUAN GONG, JIANGYAN HE, BENSHENG JU, TOONG JIN LAM, YANFEI XU AND TIE YAN

We, Zhiyuan Gong, Jiangyan He, Bensheng Ju, Toong Jin Lam, Yanfei Xu and Tie Yan declare as follows:

1. We are joint inventors of the subject matter of the above- referenced application.

2. We are submitting this declaration to demonstrate that we conceived of the claimed invention prior to March, 1998 and exercised reasonable diligence in reducing it to practice from a time just prior to March, 1998 up to the time that our Singapore priority application was filed on February 18, 1999.

3. Attached as Exhibit 1 is an application for research grant dated prior to March, 1998,[1] which lists Drs. Zhiyuan Gong and T. J. Lam as principal investigator and key team member, respectively. As can be seen from the abstract on page 2 of the document, a key aim of

[1] Note that the dates on those documents dated prior to March, 1998 have been redacted.

the project was to prepare ornamental fish for providing to the ornamental fish export industry having various fluorescence genes such as the jellyfish gene encoding green fluorescent protein (GFP). Various objectives consistent with those set forth in our patent application are described in the abstract and list of objectives on page 2. On the 7-page portion of the grant proposal identified as "Annex A," and particularly that portion entitled "Programme" beginning on page 3 of Annex A, we provided a detailed description of how this work was going to be carried out. There we disclose in some detail our proposed approach to the isolation and identification of zebrafish genes, tissue specific expression, isolation of zebrafish promoters, preparation of transgenic constructs, introduction of the transgenic DNA constructs into zebrafish, characterization of zebrafish promoter by transgenic expression and generation of stable lines of transgenic zebrafish expressing GFP.

4. Attached as Exhibit 2 is a document, also dated prior to March, 1998, which shows that the above research grant was approved and funded.

5. From the foregoing, it is evident that we had conceived of the idea of preparing transgenic fluorescent fish expressing a fluorescence gene for the purpose of providing such fish to the ornamental fish industry, as well as a method for preparing such fish, prior to March, 1998.

6. As noted in our grant proposal and repeated above, there were many facets of our ornamental fish project, not the least of which was the identification and cloning of various tissue-specific or ubiquitous promoters that could be used to express the GFP in the ornamental fish. On August 10, 1998, we submitted a manuscript for publication entitled "Fast Skeletal Muscle-Specific Expression of Zebrafish Myosin Light Chain 2 Gene and Characterization of Its Promoter by Direct Injection into Skeletal Muscle." This manuscript was subsequently published in 1999 (DNA and Cell Biology, 18:85-95; see Exhibit 3). As can be seen from

Exhibit 3, this manuscript describes in some detail the isolation and characterization of the MLC2f promoter and its testing by direct injection into skeletal muscle. This promoter is an aspect of our invention and was used by us to prepare our ornamental fluorescent transgenic fish.

7. The work that is described in the foregoing article as well as that on other promoters that we were characterizing was being conducted by us diligently in our laboratory during the timeframe beginning prior to March, 1998, leading up to the manuscript submission in August, 1998. Shown in Exhibit 4 is a collection of research notes from one of our laboratory notebooks demonstrating studies carried out from February 25, 1998 ("25/02") through April 2, 1998. (It will be noted that the page numbers appearing on various of the pages are in descending order.) These studies reflect our work in characterizing the MLC2f promoter through a deletion approach. Outward PCR was carried out to delete the proximal MEF2 (a muscle transcription activator) binding site internally in the two previously made MLC2f 5' deletion constructs with the CAT (chloramphenicol transferase) reporter gene: 79-bp and 1005-bp. These two deletion-mutation constructs were then tested together with other MLC2f 5' deletion constructs by direct injection into zebrafish muscle and we demonstrated that the mutated MEF2 site could be compensated by upstream MEF2 sites (Exhibit 3). Below we have provided a brief summary of what is shown in Exhibit 4:

A. PP. 261-256. Generation of MEF2 internal deletion in the 79-bp and 1005-bp MLC2f 5' deletion constructs:

P. 261, notes on setting up ligation to make two MLC2f internal-deletion constructs (79-bp and 1005-bp) after outward PCR and BamHI digestion that facilitated the ligation of compatible DNA ends.

P. 260, PCR reactions to ensure that the ligation was successful. One ligation was successful (79-bp construct) and the other one (1005-bp) is less successful. The 1005-bp was repeated by PCR and the result was improved but the band was still faint.

P. 259, Notes on establishing optimal PCR conditions to select internal deletions from the ligation.

P. 259-258, (March 2, 2008) preparation of CAT cloning vector by removing the MLC2f fragment from the pMLC2f-934CAT construct after HindIII and SpeI digestion.

P. 258, ligation of the 79-bp MLC2f promoter fragment to the CAT vector after internal deletion of the proximal MEF2 binding site (March 2), followed by transformation of the ligation mixture into bacterial cells (March 3).

P. 258-257. screening of bacterial colonies containing of correct DNA clones by PCR (March 4-5).

P. 257. DNA sequencing reaction to confirm the clones (March 6).

P.257-256, Similar cloning and selection of bacterial colonies for the 1005-bp internal deletion construct (March 5-11).

B. P. 255-the end. Measurement of CAT activity after injection of MLC2f deletion constructs into zebrafish muscle:

P. 255. Preparation of all MLC2f-CAT constructs for muscle injection experiments. All constructs were transformed into bacterial cells and monitored by PCR.

P. 254. Large scale of plasmid preparation for MLC2f-CAT constructs and OD reading to monitor quality of plasmid preparations.

P. 253. radioactivity reading printed directly from a scintillation counter to measure CAT activity.

P. 252, blank page.

P. 251, Calculations of CAT activity based on raw data.

P. 250, Summary of relative CAT activities for different CAT constructs at different time points and dosages.

The last page, more raw data printed directly from a scintillation counter in these experiments.

8. During this time frame we were also attempting to identify other zebrafish genes and promoters that could be used in the preparation of our ornamental transgenic fluorescent fish. One such gene was the ARP gene. Shown in Exhibit 5 are some studies relating to our isolation and characterization of the ARP gene expression and promoter. The first two pages of Exhibit 5, dated March 2-5, 1998, are notes for preparation of ARP probe (cDNA clone A150) for in situ hybridization to characterize ARP expression in zebrafish embryos. The third page shows studies dated March 23, 1998, relating to the cloning of a long ARP promoter (2.1 kb) to the pEGFP-1 vector (Clontech). The next few pages are sequence analyses of the 2.1 kb ARP promoters. The sequencing of the ARP cDNA (A150) and promoter elements, shown in the remainder of Exhibit 5, were carried out over the next several months, as evidenced by the ultimate sequencing, carried out on August 23, 1998.

9. Exhibit 6 is a collection of pages from our laboratory notebooks that evidence our work on characterization of the MCK promoter during March, 1998. These studies included the following:

P.31. The zebrafish MCK promoter was cloned into the pEGFP-1 vector and concentration of the MCK5-EGFP plasmid was determined by gel electrophoresis (March 3, 1998). In order to analyse the MCK promoter, 5' nested deletion was

carried out and the MCK5-EGFP plasmid was first cut by restriction enzymes (March 13).

P.32-33, ExoIII nuclease digestion was carried out to conduct unidirectional deletions followed by ligation (continued from March 13) and transformation (March 14). PCR was used to identify colonies with suitable deletions (March 16). Suitable colonies were inoculated for plasmid preparation (March 17-18).

P. 34, DNA concentrations of deleted MCK promoter-EGFP constructs were determined by OD reading (March 23). MCK-CAT construct was made and screened (March 25).

PP.34-37. Preparation of a series of plasmid DNAs for functional analyses and these plasmid DNAs included MCK5-EGFP, CMV-EGFP, MLCP1.2-EGFP, ARP0.8-EGFP, MCK5-CAT, MCK23-CAT (MCK5 and MCK23 are two different MCK gene promoters).

The immediate next page, raw data of CAT activities of different CAT constructs after muscle injection.

The following three pages are DNA sequence data of deleted MCK promoters (MCK5-d3, MCK5-d6 and MCK5-d7).

The last two pages are the complete sequence of the MCK promoter with indication of the start sites of deletion constructs (d3, d6. d10. d5, d12, d7 and d9).

10. Exhibit 7 displays studies and experimental notes for making 5' deletion constructs in the EGFP vector for characterization of zebrafish MLC2f and ARP promoters. The approach used here was unidirectional deletion by ExoIII nuclease, followed by ligation, transformation and PCR selection of suitable size of promoter constructs for functional analyses.

Certain of these studies were carried out from October 7, 1998 through October 16, 1998, as shown on pages 38 through 41 of Exhibit 7 as follows:

PP. 38-39, nested deletion of pMLC2kb-EGFP (Oct 7-10, 1998)

P. 40, PCR screening of colonies containing 5' deletions of MLC2 promoter (Oct 9-10).

P. 41, nested deletion of pARP-EGFP and PCR screening for deletion constructs (Oct 15-16)

The ARP promoter analysis data were published in Ju et al (1999) Dev Genetics 25:158-167. Paper submitted on Feb. 4, 1999 and accepted on March 19, 1999. (see Exhibit 8)

11. Exhibit 9 sets forth our lab notes evidencing our work on characterization of expression of muscle-specific genes in zebrafish embryos including MLC2f and MCK genes during the time frame of June, 1998 through the end of September, 1998, with some additional studies in December, 1998. This work was ultimately published in the publication of Xu *et al.*, Developmental Dynamics, 219:201-215 (2000), attached as Exhibit 10. The lab notes can be seen to evidence the following activities:

P. 60 Total RNA isolation from zebrafish embryos at different developmental stages (June 8, 1998)

PP. 61-62 Running of RNA gel (June 10), northern blot of same shown at the top of page 62

P. 62 (bottom) PCR amplification and purification of cDNA inserts from various muscle-specific clones (June 11)

P. 63 Summary of in situ hybridization results, including analysis of expression sequence of muscle genes (June 12)

PP. 64-65 Radioisotope-labeling of two muscle gene probes by the random primer approach, E371 (alpha tropomyosin) and MLC, and northern blot hybridization using the two probes (June 13-14)

P. 66-68 Re-running of RNA gel electrophoresis for northern blot experiments and re-preparation of total RNAs from zebrafish embryos of various stages (June 16-30)

P. 69 PCR amplification of desmin fragment (another muscle specific gene) and synthesis of A228 (fast muscle tropomyosin) and MLC2 probes (July 7)

PP. 70–71 Running of RNA gel for RNA blotting studies; preparation of radioactive probes to be used for probing northern blot (July 9-14)

PP. 72-73 More RNA extractions from staged embryos, running of RNA gel (July 22-23)

PP. 73-76 Preparation of non-radioactive RNA probes (DIG [dioxygenin]-labeled) for in situ hybridization and also performance of northern blot hybridization (July 24-29).

p. 77 Blank.

PP. 78-81 Random primer labeling and performance of northern blot hybridization for more muscle-specific probes, desmin, E465 (parvalbumin), E134 (troponin T) and E371 (alpha tropomyosin); photocopies of some autoradiograms of northern blot hybridization are presented in P. 81 (Aug. 3-13)

P. 82 Sequencing reaction to sequence selected muscle-specific cDNA clones (Aug. 14)

P. 83 More random primer labeling of probes E68 (Myosin heavy chain 1) and A354 (troponin C) (Aug 18)

PP. 84–85 RNA extraction from 8 hour and 10 hour embryos, running of RNA gel, labeling of probes and hybridization (Aug 21-23)

P. 86 Northern blot hybridization for desmin, E68, α-actin and MLC3 (Aug. 25-29)

PP. 87-89 Construction of an α-actin gene specific probe starting from amplification of α-actin 3' untranslated region (3'UTR), to purification, ligation and probe labeling. Simultaneously a MCK probe was also prepared by restriction digestion, purification and labeling. (Sept 2-3)

P. 87. MCK promoter was also tested by injection of MCK-EGFP construct into zebrafish embryo. (Sept 2)

PP. 90-91 Continuation of cloning of the α-actin specific probe and confirmation by PCR, and later preparation of the α-actin 3'UTR plasmid for making DIG-RNA probes for in situ hybridization. (Sept 4-10)

PP. 92-95 Preparation of fluorescent α-tropomyosin probe and DIG-RNA probes from eight muscle-specific genes and performance of both single color and double color in situ hybridization. The last two pages are summary of onset of gene expression of these muscle-specific genes based on the in situ hybridization experiments (Sept 16-21).

PP. 96-97. blank.

PP. 98-99 Summary of northern blot hybridization using these muscle-specific gene probes on different adult tissues (Dec 8-11). Some of the hybridization experiments were performed between Sept 7-11 based on the record.

12. Exhibit 11 is an application to hire a postdoctoral fellow to assist in the ornamental transgenic fish project. The application was submitted in August, 1998 and approved on August 27, 1998. This document is of additional relevance in that it describes the studies that had been carried out to date, as well as those contemplated for the future. For example, on pages

1-2 of the Exhibit 11 application, under the section entitled "Progress to date" it is noted that such progress included 1) the isolation of a few hundred zebrafish genes (cDNAs) encoding a wide range of proteins and expressed in a wide variety of tissues, which would provide a rich resource for developmental analysis and isolation of gene promoters, 2) the development of a rapid method to isolate gene promoters, including the fact that six gene promoters had been isolated to date, one from the cytokeratin (CK) gene for skin specificity, three for muscle specificity from a myosin light chain 2 (MLC2) gene and two muscle creatine kinase (MCK) gene, as well as a acidic ribosomal protein P0 (ARP) gene for ubiquitous expression, 3) demonstrated that the skin specific promoter and muscle specific promoter can direct GFP expression correctly in the respective tissues, and 4) that stable lines of GFP expressing transgenic fish are being developed. This document is also important in that it demonstrates the relevancy of the studies being described in other sections of this declaration (such as the characterization of expression of muscle-specific genes in zebrafish embryos in paragraph 11, above, and Exhibit 8).

13. Shown in Exhibit 12 is a summary sheet of DNA injections into zebrafish embryos dated from September 1998 through May 1999, involving the preparation of transgenic embryos for the purpose of testing the activity of various zebrafish promoters. This is a log sheet that our group used to record the dates that the zebrafish embryos were injected, the construct that was injected, the number of embryos that were injected, the number of embryos that survived ("S") and expressed ("E") the GFP at various timepoints post-injection, the tissue specificity of expression and remarks regarding the level of expression. On the first page is recorded 16 separate experiments carried out between September 6, 1998 and February 4, 1999, involving the use of the ARP promoter ("ARP"), the MCK promoter ("MCK"), the MLC (or

MLC2, MLC2f and MYLZ2) promoter ("MLC") and the CKP (or CK) promoter ("CKP"). As can be seen, many if not most of these studies resulted in embryos that survived and expressed the GFP at the 48 hour time point. Shown on the second page of Exhibit 13 is a similar log of 5 studies carried out during the month of October, 1998. The last page of Exhibit 13 shows 11 injection studies carried out in May, 1999. During the interim times between injections, the injected embryos were grown up and germline transmission of the transgenes was screened for selection of stable transgenic lines. We were also busy analyzing the data from previous injection experiments and planning further studies carried out in connection with subsequent injection experiments as well as other relevant experiments such as characterization of muscle-specific expression in zebrafish embryos.

14. Exhibit 12 is a second grant application on transgenic ornamental fish entitled "Production of fluorescent transgenic ornamental fish." The grant application was submitted on February 1, 1999, and funding was requested for additional developmental work on the invention. As can be seen from section II. (i) of the Annex A, this document briefly reviews the work that had been accomplished up until that time on the fluorescent transgenic ornamental fish project. It is stated that transgenic ornamental fish should be more acceptable to regulatory agencies and consumers, and proceeds to outline the approach that we took in the preparation of our ornamental fish, including the use of a gene encoding GFP under the control of a tissue-specific or a ubiquitous promoter (work that is reflected hereinabove). The application then proceeds to note that we had prepared as of that time four GFP transgenic constructs (pCK-EGFP, pMCK-EGFP, pMLC2f-EGFP and pARG-EGFP), and that when these chimeric gene constructs were introduced into fish, all of them showed predictable expression patterns according to the specificities of the promoters used. It is then stated that a patent for the constructs was being

filed. (The foregoing is described in the second page of Annex A of Exhibit 12). In the paragraph bridging the second and third pages of Annex A, it is further stated that we intended to isolate additional gene promoters that would permit targeting transgene expression in any tissue, and that we contemplated extending our work in zebrafish to other ornamental fish such as medaka, goldfish, koi, carp and glass catfish. At the bottom of the third page and top of page 4 of Annex A, many additional aspects of the present invention are explained, including, for example, the development of skin-specific, muscle-specific and ubiquitously expressing fish, and the use of other colors and mixture of colors. Further pages of Annex A include additional details regarding the preparation of other types of transgenic ornamental fish.

15. From the foregoing evidence, it is quite clear that we had conceived of our invention prior to March, 1998 and were diligent in reducing the present invention to practice during the time frame of just prior to March, 1998 through our Singapore filing date of February 18, 1999. The following table is a brief summary of our activities during this period.

Period	Activities	Remarks
Prior to March, 1998 (Conception)	Applied for and obtained our first research grant from National University of Singapore to support transgenic ornamental fish research and the title of the project is "Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent	Exhibit 1, 2

	protein (GFP)". This document demonstrates both that we had the idea and had a detailed understanding on how to carry it out.	
March 1998 to April 2, 1998	Characterization of zebrafish MLC2f promoter by direct injection of deletion constructs into zebrafish muscle	Exhibits 3 and 4
March 2, 1998 to August 23, 1998	Characterization of zebrafish ARP gene expression and promoter	Exhibit 5
March, 1998	Characterization of zebrafish muscle-specific MCK promoter	Exhibit 6
Aug. 1998	Preparation and submission of grant proposal to support a postdoctoral fellow to work on the fluorescent transgenic ornamental fish project. The grant was approved on Aug. 27, 1998	Exhibit 11

Oct 7-16, 1998	Preparation of 5' deletion constructs for zebrafish MLC2f and ARP promoters.	Exhibit 7
June 8, 1998 – September 21, 1998, and December, 1998	Characterization of expression of muscle-specific genes in zebrafish embryos including MLC2F and MCK genes	Exhibits 9 and 10
Sept 6, 1998-Feb 4, 1999	Microinjection of zebrafish promoter-GFP constructs	Exhibit 12
Jan 1999	Preparation and submission of the second research grant application on production of transgenic ornamental fish. The grant was submitted to National University of Singapore and entitled "Production of fluorescent transgenic ornamental fish (submitted on Feb. 1, 1999)	Exhibit 13

16. We hereby declare that all statements made of our own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date Feb. 15, 2008	/Zhiyuan Gong/
Date	Jiangyan He
Date Feb.14, 2008	/Bensheng Ju/
Date	Toong Jin Lam
Date	Yanfei Xu
Date	Tie Yan

imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

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Date Bensheng Ju

Date Feb. 15, 2008 / / Toong Jin Lam/

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Date	Tie Yan

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Date Feb. 15, 2008 /Zhiyuan Gong/

Date Feb.15, 2008 /Jiangyan He/

Date Bensheng Ju

Date Toong Jin Lam

Date Yanfei Xu

Date Tie Yan

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Date Feb. 15, 2008	/Zhiyuan Gong/
Date	Jiangyan He
Date	Bensheng Ju
Date	Toong Jin Lam
Date	Yanfei Xu
Date Feb. 19, 2008	/Tie Yan/

EXHIBIT 1

Reference number: _____ Form RUR/RG1

Multi-Disciplinary Research : Yes / No + (Delete where appropriate)

NATIONAL UNIVERSITY OF SINGAPORE
ACADEMIC RESEARCH FUND
APPLICATION FOR A RESEARCH GRANT

TO: THE UNIVERSITY RESEARCH COMMITTEE/
THE ACADEMIC RESEARCH FUND COMMITTEE/
THE MINISTERIAL LEVEL COMMITTEE
(Delete where appropriate)

1 PRINCIPAL INVESTIGATOR Name: Dr. Zhiyuan Gong Employee number: 1125H Appointment: Lecturer Department: Zoology Tel: 7722860 Fax: 7792486 Previous grants from Academic Research Fund: , RP950304, $223,335	Attach 1-page C.V. of Principal Investigator, giving an outline of education and work experience, track records in managing research projects and the number of international journal and conference papers. Also list selected relevant publications (not more than ten). To state the date/amount of previous grants.
2 * COLLABORATOR(S)/OTHER KEY TEAM MEMBERS Name: Prof. T.J. Lam Employee number: 00706G Appointment: Head and Professor Department: Zoology Tel: 7722692 Fax: 7792486 Previous grants from Academic Research Fund:	To provide details for each collaborator/key team member. Attach 1-page C.V. of each member, giving an outline of education and work experience, track records in managing research projects and the number of international journal and conference papers To state the date/amount of previous grants.

* Please use a separate sheet if there is insufficient space and attach it to this form.

+ Defined as any research that requires input from staff in a different department or staff belonging to other disciplines from other institutions outside the University.

3 TITLE OF RESEARCH PROJECT	Title should be short and concise.
Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP)	
4 ABSTRACT Ornamental fish is an important export industry in Singapore. In the present grant application, we propose to use a modern transgenic technique to generate novel varieties of ornamental fish by incorporation and expression of a jellyfish gene coding for green fluorescent protein (GFP). During the course of this work, a rapid cDNA clone tagging approach, or sequencing randomly selected clones by single run sequencing reactions, will be used to isolate and identify zebrafish genes in bulk. Interesting promoters will be isolated based on the sequence information from these tagged cDNA clones and characterized by transient expression in transgenic zebrafish. Useful promoters will be selected to generate stable lines of GFP transgenic zebrafish. The initial phase of this research is to focus on the following 5 patterns of GFP expression in transgenic zebrafish: ubiquitous expression, muscle specific expression, skin specific expression, heat inducible expression and heavy metal inducible expression.	In about 200 words, describe the project in the context of previous work done or in progress at the University or at other institutions, and explain the uniqueness of this approach.
5 LIST MAIN OBJECTIVES IN ORDER OF PRIORITY 1. Bulk isolation and identification of zebrafish genes by cDNA clone tagging. 2. Isolation of selected tissue-specific and inducible zebrafish promoters. 3. Characterization of the zebrafish promoters by transient expression in transgenic zebrafish. 4. Development of stable lines of green fluorescent transgenic zebrafish with different promoters. After completion of the project, there should be a few high quality papers suitable for publication in high profile international journals. Many zebrafish genes will be isolated and characterized to facilitate future studies in zebrafish molecular biology. The stable lines of GFP transgenic zebrafish can be explored commercially.	Describe the objectives clearly and succinctly, and highlight the deliverables upon project completion. Attach a self-contained case for support, consisting of no more than 6 A4 pages. Some assistance in preparing of this is given in Annex A.

6 POTENTIAL APPLICATIONS/EXPLOITATION	State the likely applications of the work (technological, social, scientific, economic). Also explain any exploitation potential, and the follow-up arrangements that would be required.
Stable lines of GFP-transgenic zebrafish will be marketable as new exotic fish. The gene resource explored in zebrafish can be applied to other fish species, both ornamental and food fish species. The transgenic technique developed in this study will also be applicable to other transgenic research with important economic implication, such as increase of growth rate, disease resistance and sex reversal etc.	
7 COLLABORATIONS	Where appropriate, describe any collaborative arrangements, including arrangements for exploitation and protection of intellectual property.
Prof. Choy L. Hew, U. of Toronto, Canada, will be collaborating this research and spend his 3-6 month sabatical with us in	

8 SUMMARY OF RESEARCH GRANT REQUESTED

Grant requested must cover the entire project life. Applicants should note that research grant, once approved, will not be increased except for salary increases of manpower.

	Year 1	Year 2	Year 3 *	Total ($)
Manpower	31,400	33,600	36,200	101,200
New equipment/facilities	9,000	0	0	9,000
Materials/consumables	21,500	21,500	21,500	64,500
Training/other misc. costs	1,200	1,200	1,200	3,600
Grant Total ($)	63,100	56,300	58,900	170,300

* Please use a separate sheet if project life is expected to exceed 3 years. The Total column should reflect the total grant required for the entire project life.

Please see Notes for Budget Preparation in Annex B for assistance in completing items 8.1 to 8.4.

8.1 MANPOWER COSTS (for additional staff only)

Please indicate an "F" against the number if it is a continuation of an existing appointment.
NS increments* – Please tick against staff grade if increments are to be given for National Service (NS).

Manpower	Staff Grade	With NS*	Number		Annual Cost ($)			No. of Months on Project	Total Cost ($)
			Full Time	Part Time	Year 1	Year 2	Year 3		
Research Assistant									
Technician/Jr Research Assistant	LT (pass)	No	1		31,400	33,600	36,200	36	101,200
Student Assistant									
Research Scholar									
Research Student									

3(i)

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8.2 NEW EQUIPMENT/FACILITIES COSTS

Item Description	Unit Price ($)	Quantity	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
Microinjector	9,000	1	9,000			9,000
Grand Total ($)			9,000			9,000

8.3 COSTS OF MATERIALS/CONSUMABLES

Item Description	Unit Price ($)	Quantity	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
Molecular reagents	200	120	8,000	8,000	8,000	24,000
Radioisotopes	300	30	3,000	3,000	3,000	9,000
Chemicals	100	90	3,000	3,000	3,000	9,000
Glassware	40	180	2,400	2,400	2,400	7,200
Oligonucleotides	80	60	1,600	1,600	1,600	4,800
Film & Pictures			2,000	2,000	2,000	6,000
Fish & Feeding			1,500	1,500	1,500	4,500
Grand Total ($)			21,500	21,500	21,500	64,500

8.4 TRAINING/OTHER MISCELLANEOUS COSTS

Item Description	Purpose	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
Taxi fare	live fish transportation	200	200	200	600
Miscellaneous	slide, photocopy	1,000	1,000	1,000	3,000
	courier, stationary etc.				
Grand Total ($)		1,200	1,200	1,200	3,600

* Please use a separate sheet if project life is expected to exceed 3 years. The Total column should reflect the total grant required for the entire project life.

9. OTHER SOURCES OF FUNDING	
Name and address of other funding parties: Contact name: Contact number:	
Type of organisation: (eg industry, commerce, research institutes, government etc)	
Details of contribution: Cash: Equipment/materials: Staff secondment: Facilities: Others: Total value of funding ($) :	

10 PROJECT IMPLEMENTATION SCHEDULE

Quarters\Research milestones	Year 1				Year 2				Year 3			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
1.clone tagging	X	X	X	X	X	X						
2.gene promoters				X	X	X	X	X				
3.transient expression						X	X	X	X	X		
4. stable lines							X	X	X	X	X	X

Estimated start date:	The start date is defined as the first date on which the project commits or incurs expenditure. Researchers are reminded that a project, once approved, must start within 60 days of approval.
Estimated completion date: 06/99	

11 DECLARATION

We declare that the facts stated in this application and the accompanying information are true.

	Signatures and dates	
	Principal Investigator	Collaborating party (if any)
Applicant(s) : Dr. Zhiyuan Gong Prof. T.J. Lam		
Endorsed by: (1) Head of Department (2) Chairman, Faculty Research Committee (3) Director of Research (4) Chairman, University Research Committee OR Chairman, Academic Research Fund Committee		

12 HEAD'S COMMENTS :

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

Signature Date

13 FACULTY RESEARCH COMMITTEE'S COMMENTS :

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

Signature Date

14 DIRECTOR OF RESEARCH'S COMMENTS :

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

Signature Date

ANNEX A

I. PURPOSE

Singapore is the world largest exporter in ornamental fish with an export value of $78.5 million dollars in 1994 (Department of Statistics, Singapore 1994, 1995). To maintain the leadership in this competitive industry, it is necessary to continuously produce new varieties with novel shapes and color patterns. Traditional approaches to create new varieties are genetic breeding and selection, but these approaches are rather slow and unpredictable. The use of color dyes in many pet stores is both temporary and unsatisfactory. In the present grant application, we propose to use a modern transgenic technique (1-3) to generate new varieties of ornamental fish with distinct and predictable color patterns.

As an initial step to explore the suitability of the transgenic technique to ornamental fish, zebrafish is selected as a model. Zebrafish is one of the most popular ornamental species and also increasingly an important model for developmental biology (4). As an experimental model, zebrafish offers many advantages, e.g. external and transparent embryogenesis, easy availability and low cost to maintain, being a good model in both genetics and embryology research, etc. Because of these advantages, zebrafish has increasingly attracted attentions around the world in the past few years. The cell lineage of embryogenesis has been established (5), thousands of mutants has been created by saturated mutation (6,7), and a genetic linker map has been constructed by RAPD-PCR (8). Transgenic zebrafish has also been reported (9-12). However, the genes transferred in all cases are derived from other species such as virus and mammals due to the lack of the molecular resource from zebrafish. The number of zebrafish genes isolated, compared to other well established model organisms such as the fruit fly and mouse, is rather limited. In the present proposal, we intend to expand the repertoire of the gene pools by isolation of large number of zebrafish genes and selected promoters. These include: 1) rapid isolation and identification of zebrafish genes by cDNA clone tagging (13); 2) selected isolation and characterization of zebrafish gene promoters; 3) tissue specific expression of reporter gene in transgenic zebrafish to test the suitability of the promoters; and 4) generation of distinct color patterns by transgenic expression of green fluorescent protein (GFP).

Five types of transgenic zebrafish will be produced in the present proposed research: 1) ubiquitous green: green color is produced in all tissues by expression of GFP under a strong and ubiquitous promoter; 2) green muscle: green color in muscle under a muscle specific promoter; 3) green skin: green color in skin under a skin specific promoter; 4) heat-inducible green: green color is produced at high temperature under a heat shock promoter; and 5) metal inducible green: green color is produced by addition of heavy metals such as zinc and cadmium under a metal inducible promoter. The resource explored in zebrafish will also be useful and applicable to other ornamental fish species. The genetically altered ornamental fish will be marketable.

II. BACKGROUND

(i) Previous work

The basic transgenic approach to create novel varieties of ornamental fish is to insert a gene encoding a color protein into the genome of selected fish species under a selected promoter. The selected promoter will direct the color protein to express in certain tissues or under certain induction conditions such as heat shock and heavy metals; thus, new color patterns will be created. The color gene chosen in the present proposal is green fluorescent protein (GFP) gene which was isolated from a jelly fish (14). GFP emits green light under blue and ultra violet light without the need of substrate. GFP has no adverse effect to cellular activity and thus can be used in live organisms. Transgenic expression of GFP has

been reported in nematode and fruit fly (15,16). Recently, the feasibility of expression of GFP has also been demonstrated in zebrafish (17); however, thus far, all studies on transgenic zebrafish utilized heterologous promoters derived either from virus or from other species. The effectiveness of transgenic expression is frequently limited by these heterologous promoters (3).

In order to generate successful transgenic zebrafish for ornamental application as well as developmental analysis, it is necessary to isolate more zebrafish genes and promoters. It is generally accepted that a homologous promoter is preferably used as compared to a heterologous promoter. Currently, the number of zebrafish genes isolated is rather limited and no zebrafish promoter has been reported. Therefore, there is an urgent need to increase the availability of zebrafish genes and promoters to improve the infrastructure studies in zebrafish. The traditional method to isolate a gene is screening of a gene library, which is slow, labor intensive and expensive. We have adapted a new method to rapidly isolate and identify zebrafish genes. This method is based on the work in human genome project to sequence random cDNA clones by a single run reaction and the sequences obtained by this methods are called expressed sequence tags (EST's) (18). Briefly, we sequence randomly selected cDNA clones from a cDNA library by a single sequencing reaction to obtain 200-300 base pairs of sequences for each clone. These sequences are then used as tags for these clones to search for homology in DNA databases. In this way, about 40% of the random clones sequenced can be identified based on sequence similarity. Previously, we have found that this is a highly efficient way to increase the repertoire of cloned fish genes (13). Since then, we have accumulated hundreds of random clones from two zebrafish cDNA libraries: an embryonic library and an adult library. Over 100 zebrafish genes have been identified and many of them can be predicted to be expressed in a specific tissues (Lee and Gong, unpublished data). The expression patterns of some of these genes have been confirmed by in situ hybridization.

Interesting clones identified can then be used to isolate gene promoter. For example, one of the clones identified encodes myosin light chain 2 (MLC2) which is specifically expressed in skeletal muscle. This was confirmed by in situ hybridization (He and Gong, unpublished data). To isolate a muscle specific promoter, we have developed a rapid method based on a linker mediated polymerase chain reaction (PCR). In this method, a synthetic oligonucleotide linker, which has a dideoxynucleotide in one strand to prevent non-specific amplification, is ligated to genomic DNA after complete restriction digestion. Promoter region is then amplified by nested PCR using gene specific primers derived from 5' end of the cDNA clone and primers from the synthetic linker. By this method, we have successfully isolated an 1.1 kilobase MLC2 promoter which contains several obvious muscle specific elements (Chan and Gong, unpublished). Other cDNA clones currently available from our tagged cDNA clones to isolate tissue specific promoters include heart α-actin (heart), crystallin (eye), vitellogenin (liver), cytokeratin (skin) etc.

(ii) Research experience

In the present proposal, many state-of-the-art techniques in modern molecular biology will be used. These techniques include molecular cloning, DNA sequencing, polymerase chain reaction (PCR), in situ hybridization, microinjection and electroporation etc. The principle investigator, **Dr. Z. Gong**, have been actively involved in molecular biology research for the past 12 years and have hand-on experience on all techniques required in the proposed research. His major research activities include gene regulation in sea urchin embryos, characterization of fish gene promoters, cloning and expression of pituitary hormone genes, structure and function of antifreeze proteins, transgenic fish, and fish homeobox genes. ***He has published 5 papers on transgenic fish in international journals in the past 5 years.*** Currently, his research group consists of one full time research assistant who works on my homeobox gene project, and one graduate student who works

on molecular vaccine for fish disease. Both of them are now well trained in molecular biology and can supervise newcomers for most of these techniques required for the proposed research project. The co-investigator, **Prof. T.J. Lam**, has 31 years of research experience in biological research on fish and is a prominent scientist in this field.

III. PROGRAMME

1. Isolation and identification of zebrafish genes

In order to further increase the repertoire of tagged zebrafish cDNA clones to construct a tagged cDNA library which is a collection of tagged cDNA clones, we propose to continue to sequence randomly selected cDNA clones. To maximize the representation of all expressed genes, two cDNA libraries were constructed: one is an embryonic cDNA library which was made with a mixed stages of zebrafish embryos, and the other an adult cDNA library which are made from a mixed male and female fish. cDNA clones will be selected randomly for sequencing and the sequence information will be used to identify more zebrafish genes and thus to expand the availability of zebrafish gene resource. Tissue specific cDNA clones identified by this approach will be used for isolation of their promoters. These tagged clones can also be used for physical mapping, tissue and cell type marker, investigation of gene expression, and expression of useful proteins etc.

2. Tissue specific expression

Selected clones with presumptive tissue specificity will be confirmed by in situ hybridization. The cDNA clones isolated are in pBluescript vector which contain T3 and T7 promoters to generate sense and antisense riboprobes respectively. DIG-UTP labeled riboprobes will be used for whole mount in situ hybridization on paraformaldehyde fixed embryos and fry, and anti-DIG antibody conjugated with alkaline phosphatase will be used for color development in the presence of substrate (19).

3. Isolation of zebrafish promoters

Selected cDNA clones will be used for promoter isolation. These clones will be sequenced completely for design of gene specific primers. The promoters will be isolated by our newly developed linker mediated PCR method. In the present proposal, the following five gene promoters for transgenic research will be concentrated on:

1) Ubiquitous promoter: The promoter will be isolated using an elongation factor 1α cDNA clone. EF1α is an essential protein factor in translational machinery and are present in all cell types. EF1α mRNA is a highly abundant species in all cells and its cDNA clone constitutes a few percentage of clones in the two zebrafish cDNA libraries we constructed (our unpublished data); thus, the gene for EF1α likely has a strong promoter to function ubiquitously in all tissues. EF1α cDNA clone has already been identified in our tagged cDNA library and will be sequenced completely to design PCR primers for isolation of its promoter by our linker mediated PCR.

2) Muscle specific promoter: Muscle is the largest tissue in adult fish and muscle specific gene expression is the easiest to characterize. A myosin light chain 2 promoter has been isolated and will be characterized by direct injection into muscle (20) and by transient expression in transgenic zebrafish (see Sections 4-5).

3) Skin specific promoter: Skin is a surface tissue and the transgene expression can be conveniently detected. Most color patterns in ornamental fish is due to the pigmentation in skin cells. A skin specific cDNA clone encoding cytokeratin has been isolated by cDNA clone tagging and will be used to isolate this type of promoter.

4) Heat shock promoter: Heat shock promoter is useful to conditionally express a transgene. The transgene will only be expressed under heat shock condition. A heat shock promoter can be isolated from a heat shock protein cDNA clone. Several heat shock protein cDNA clones have been isolate from our tagged clones and will be used for isolation of a heat shock promoter.

5) Metal inducible promoter: This type of promoter can be induced to express to a high level by heavy metals such as zinc and cadmium. The best characterized metal inducible promoter is metallothionein gene promoter. Zebrafish metallothionein cDNA clone will be isolated by hybridization screening using a homologous probe from winter flounder (13) if we can not obtain a suitable clone by cDNA clone tagging. The promoter will be isolated based on the metallothionein cDNA sequence.

4. Transgenic DNA constructs

In order to characterize the isolated promoters and confirm their tissue specificity in vivo, the promoter fragments will be ligated to report genes to be used in transgenic research. The following three reporter genes will be used in the present study:

1) CAT gene: CAT is chloramphenicol acetyltransferase which can be used to acetylate ^{14}C-chloramphenitol and its activity can be easily quantitated from the tissue lysate. Thus, CAT assay is important to analyze the promoter activity (21).

2) Lac Z gene: lac Z encodes β-galactosidase which is highly sensitive and can be used to locate the transgene expression in situ in the presence of the substrate X-gal. Thus it is important to locate tissue specificity and more sensitive than the GFP system.

3) GFP gene: Detection of GFP requires no substrate and thus can be performed in live transgenic fish. But the detection sensitivity is lower than lacZ reporter. These constructs will be eventually used to develop stable transgenic lines for ornamental purpose.

5. Introduction of transgenic DNA constructs into zebrafish

The methods to introduce a foreign DNA into zebrafish include microinjection (9), electroporation (10), retroviral infection (11) and sperm mediated transfer (12). In the proposal, we will try microinjection and electroporation. So far, microinjection is the most reliable method to deliver foreign DNA but this method is tedious and requires highly skillful personnel to perform. Electroporation has also been reported to be successful and it can electroporate a few hundred eggs in a short period. However, this method is less efficient and the electroporation conditions need to be optimized. In the present research, we will try to develop an optimal electroporation condition for generation of transgenic zebrafish.

6. Characterization of zebrafish promoter by transient expression

The activity of these promoters will be tested and compared by transient expression studies in transgenic zebrafish. The transgenic DNA constructs will be introduced into the egg and the expression of the transgene will be examined in early embryogenesis and hatched fry. The level of expression can be monitored by CAT gene construct and tissue specificity by Lac Z gene constructs. To monitor the expression of a transgene in live fish, GFP gene constructs will be used. For heat shock and metal inducible promoters, corresponding inducible conditions will be applied to monitor the expression of the transgenes. Sometimes deletion of certain promoter regions may be necessary to ensure an optimal activity.

7. Generation of stable line of transgenic zebrafish expressing GFP

The ultimate purpose in the present proposal is to generate novel color patterns of transgenic fish. Once the effectiveness of these promoters is confirmed by above studies, stable lines of GFP transgenic zebrafish with the five different promoters will be established. To establish stable lines of transgenic fish, zebrafish embryos with the

introduced GFP gene will be raised to adulthood and transgenic adult will be screened by PCR using a piece of fin tissue. The expression of GFP gene may be a visible phenotype in transgenic zebrafish and can be monitored with a non-invasive approach by maintaining the fish under a blue or ultraviolet light. The transgenic adult with a demonstrated expression of GFP gene will be used to cross a non-transgenic individual to obtain second generation of transgenic fish and the positive individual will be crossed with another positive individual to obtain both heterozygotes and homozygotes. The stable line of transgenic fish is usually obtained after the second generation. The economic value of these transgenic fish will be explored at this stage. Other interesting fluorescent patterns of GFP transgenic zebrafish will be generated by using different promoters depending on the types of cDNA clone obtained by cDNA clone tagging. The gene resource obtained from the zebrafish will be applied to other ornamental fish species.

IV. RESOURCE

(i) Manpower Cost

The project is labor intensive. I propose to hire one full time research assistant at the level of **LT (Grade B) Pass with merit** throughout the three years of project. Currently I have a full time LT who works on my other project (RP950304). He is fully occupied with the funded project as well as maintenance of the laboratory and aquarium. Extra manpower is needed to initiate the new project.

(ii) New Equipment/Facilities costs

Majority of the laboratory equipment has been provided by my first grant (RP950304) and will be shared by the new project. Some instruments such as electroporator and microscopes are already available in our department and will not be requested. In the present grant, we only request one item which is essential for the present project.

1) Microinjector: This will be used for the delivery of transgenic construct into the egg and is needed routinely throughout the proposed project. Because of the high demand of this instrument in the present project, it is impossible to use the microinjector in other laboratories which can not accommodate our need.

(iii) Materials and Consumables:

The items of consumables are listed in section 8.3.

(iv) Miscellaneous costs

These include taxi fare for transportation of live fish ($600) and miscellaneous costs including preparation of diagram and slide for publication, courier expense for exchanging experimental materials, and photocopy and stationary etc.($3,000).

(v) International collaboration

Dr. Choy L. Hew, Professor of Biochemistry at the University of Toronto, Canada, is an internationally renown expert in transgenic fish research and his Canadian team reported the first successful growth hormone transgenic salmon with a dramatic enhancement of growth rate (2). He is proposing to spend 3-6 month sabbatical in my laboratory in 1997 and is interested in the proposed project. This is an exceptional opportunity to enhance our transgenic research of the proposed project. During the course of the project, we will contact each other to exchange experimental data and materials. We will benefit from the collaboration to acquire experimental materials and protocols, to learn new techniques and to keep track of the progress in the field of transgenic research.

A copy of confirmation letter from Prof. C.L. Hew is attached.

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Curriculum Vitae: Dr. Zhiyuan Gong

Education:

BSc (1982) Ocean University of Qingdao, China
Ph.D (1987) McGill University, Canada

Work Experience:

1987-1988: Postdoctor, McGill University, Canada
1988-1995: Postdoctor and Research Fellow, Hospital for Sick Children, Toronto, and University of Toronto, Canada
1995-present: Lecturer, National University of Singapore, singapore

Research Experience:
Research grant managed in NUS:

1. Principal Investigator: Developmental regulation and functional analysis of a family of LIM domain homeobox genes in zebrafish (RP950304).

2. (Transferred from Dr. Xu Guo-Liang) Co-investigated with A/P Y.M. Sin and Prof. T.J. Lam, Identification of surface antigens in *Ichthyophthirius multifiliis* and the development of fish vaccine. (RP954346).

Publications:

Number of papers in international journals: 25.
Number of conference papers: 22.

10 Selected and Recent Publications:

*1. Goug, Z., C.L. Hew, and I.R. Vielkind (1991) Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese Medaka embryos. *Mol. Marine Biol. Biotech.* **1**: 64-72.

*2. Du, S.J., Z. Gong, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler, and C.L. Hew (1992) Growth enhancement in transgenic Atlantic salmon by use of fish antifreeze/growth hormone chimeric gene constructs. *Bio/Technology* **10**:176-181.

*3. Du, S.J., Z. Gong, C.H. Tan, G.L. Fletcher and C.L. Hew (1992). The design and construction of "all fish" gene cassette for aquaculture. *Mol. Marine Biol. Biotech.* **1**:290-300.

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*5. Gong, Z., and C.L. Hew (1995) Transgenic fish in aquaculture and developmental biology. *Current Topic in Developmental Biology.* **30**:175-213.

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* Transgenic fish papers

EXHIBIT 2

NATIONAL UNIVERSITY OF SINGAPORE

DATE:

DR GONG ZHIYUAN

DEPT OF ZOOLOGY

NUS

ACADEMIC RESEARCH GRANT FOR FY 96

I am pleased to inform you that your application for research grant has been approved. The approved budget for the project and the Term & Conditions of the grant are attached.

The procedures and Terms & Conditions for research grants have been revised substantially with effect from FY 94 and you are advised to refer to the circulars from Director of Research, ref. BUR/94059, BUR/94097, and BUP/94111 on the Revised Procedures for Research Grants, copies of which are obtainable from your Head of Department.

Your attention is drawn to paragraph 2 of the Terms & Conditions of the grant on the requirement to adhere strictly to the approved budget. Please also take note of paragraph 11 regarding the submission of annual appraisal report to the Dean. Failure to submit the report by 15 March each year will result in immediate termination of funding.

Kindly return the Research Grant Acceptance Form BUR/RG3 within 14 days of the actual start date of the project. The actual start date would be the date of the first purchase order issued, or the date of invitation to tender or the date that Personnel Department is requested to commence staff recruitment, whichever is earlier. Your project must start within 60 days from the date of this approval letter, otherwise the grant may lapse.

Should you need further clarification, please contact me at extension 6344.

Yeo Seow Leng (Miss)
for Bursar

C.C. DEAN
FACULTY OF SCIENCE

C.C. HEAD
DEPT OF ZOOLOGY

RESEARCH COMMITTEE: BIOLOGICAL SCIENCES
FACULTY : SCIENCE
DEPARTMENT : ZOOLOGY
P. INVESTIGATOR 1 : DR GONG ZHIYUAN

PROPOSAL NO : B011 (RP960632)
PROJECT NO : RP960315
PROJECT TITLE : GENERATION OF NOVEL VARIETIES OF ORNAMENTAL FISH BY TRANSGENIC EXPRESSION OF GREEN FLUORESCENT PROTEIN (GFP)

ITEM	DESCRIPTION	$	AMOUNT APPROVED $
	EQUIPMENT / FACILITIES COST		9,000
1	MICROINJECTOR	9,000	
	COST OF MATERIALS / CONSUMABLES		13,498
2	MOLECULAR REAGENTS	5,000	
3	RADIOISOTOPES	1,833	
4	CHEMICALS	1,833	
5	GLASSWARE	1,500	
6	OLIGONUCLEOTIDES	1,000	
7	FILM & PICTURES	1,233	
8	FISH & FEEDING	933	
9	SLIDE, PHOTOCOPY, COURIER & STATIONERY	166	
	AMOUNT APPROVED FOR		22,498
	MANPOWER		31,400
10	LAB TECHNICIAN (GRADE B-PASS WITH MERIT)	31,400	
	COST OF MATERIALS / CONSUMABLES		13,499
11	MOLECULAR REAGENTS	5,000	
12	RADIOISOTOPES	1,833	
13	CHEMICALS	1,833	
14	GLASSWARE	1,500	
15	OLIGONUCLEOTIDES	1,000	
16	FILM & PICTURES	1,233	
17	FISH & FEEDING	933	
18	SLIDE, PHOTOCOPY, COURIER & STATIONERY	167	
	AMOUNT APPROVED FOR ..		44,899
	MANPOWER		33,600
19	LAB TECHNICIAN (GRADE B-PASS WITH MERIT)	33,600	
	COST OF MATERIALS / CONSUMABLES		13,593
20			
21	MOLECULAR REAGENTS		
22	RADIOISOTOPES		
23	CHEMICALS		
24	GLASSWARE		
25	OLIGONU		

21	RADIOISOTOPES		
22	CHEMICALS	1,834	
23	GLASSWARE	1,834	
24	OLIGONUCLEOTIDES	1,500	
25	FILM & PICTURES	1,000	
26	FISH & FEEDING	1,234	
27	SLIDE, PHOTOCOPY, COURIER & STATIONERY	934	
		167	
	AMOUNT APPROVED FOR		47,103
	TOTAL :		114,500

EXHIBIT 3

DNA AND CELL BIOLOGY
Volume 18, Number 1, 1999
Mary Ann Liebert, Inc.
Pp. 85–95

Fast Skeletal Muscle-Specific Expression of a Zebrafish Myosin Light Chain 2 Gene and Characterization of Its Promoter by Direct Injection into Skeletal Muscle

YANFEI XU, JIANGYAN HE, HO LIAN TIAN, CHIEW HUA CHAN, JI LIAO, TIE YAN, TOONG JIN LAM, and ZHIYUAN GONG

ABSTRACT

A zebrafish myosin light chain 2 cDNA clone was isolated and characterized. Sequence analysis of the clone revealed a high homology with the mammalian and avian genes encoding the fast skeletal muscle isoform, MLC2f. *In situ* hybridization and Northern blot hybridization analyses indicated that the zebrafish *MLC2f* mRNA is expressed exclusively in the fast skeletal muscle. Ontogenetically, the *MLC2f* mRNA appears around 16 hours postfertilization (hpf) in the first few well-formed anterior somites. At later stages, the *MLC2f* mRNA can also be detected in fin buds, eye muscles, and jaw muscles. To develop a useful model system for analyzing muscle gene regulation, the promoter of the zebrafish *MLC2f* gene was isolated and linked to the chloramphenicol acetyltransferase (CAT) reporter gene. The MLC2f/CAT chimeric constructs were analyzed by direct injection into the zebrafish skeletal muscle, and significant CAT activity was observed; in contrast, little or no CAT activity was generated from a similarly injected prolactin gene promoter/CAT gene construct. Within the 1 kb of the *MLC2f* promoter region, several MEF2-binding sites and E-boxes were identified, suggesting that *MLC2f* can be regulated by muscle transcription factors MEF2 and myogenic bHLH proteins. A 5′ deletion analysis indicated that the proximal 79 nucleotides from the transcription start site, which contains a single MEF2-binding site, is sufficient to drive a high level of CAT activity in injected muscle. Internal deletion of the MEF2 element in the −79-bp construct caused an 80% decrease in CAT activity, whereas internal deletion of the same MEF2 element in a −1044-bp construct had no effect on induced CAT activity. These observations suggest that an MEF2 element is important to activate the *MLC2f* gene in muscle cells, and the effect of loss of the proximal MEF2 element can be compensated for by the presence of the upstream MEF2 elements. This study also demonstrated that direct injection of DNA into skeletal muscle is a valid and valuable approach to analyze muscle gene promoters in the zebrafish.

INTRODUCTION

MYOSIN IS A COMPLEX multimeric protein that plays a central role in contractile processes in eukaryotes. Each molecule of the protein consists of two myosin heavy chains (MHC) and two pairs of myosin light chains (MLC). The light chains exist in two forms: the alkali chains (MLC1/3, nonphosphorylatable) and the regulatory chains (MLC2; phosphorylatable) (for review, see Emerson and Bernstein, 1987), both including multiple isoforms which are expressed differentially in different cells or in the same cell at different stages of development (Parker *et al.*, 1985; Kumar *et al.*, 1986). In vertebrates, the *MLC2* gene family includes at least three unique genes: one expressed in fast skeletal muscle (*MLC2f*), the second in cardiac

Department of Biological Sciences, National University of Singapore, Singapore.

and slow skeletal muscle (*MLC2s*), and the third in smooth muscle and nonmuscle cells (Shani, 1985; Kumar *et al.*, 1989; Lee *et al.*, 1992). Different combinations of MLC2 isoforms are expressed in different muscles in a developmentally regulatory and muscle-specific manner. These attributes make *MLC2* genes an excellent model system for studies of muscle-specific gene expression as well as of differential expression of multigene families during development.

Whereas the structure, function, and regulated expression of different isoforms of *MLC* genes have been relatively well characterized in the mammalian and avian systems, little is known about the *MLC* genes in lower vertebrates such as teleost fishes. In recent years, the zebrafish has become an increasingly important vertebrate model for developmental and genetic analysis (Kimmel *et al.*, 1988; Driever *et al.*, 1994). As a model organism, the zebrafish has several advantages; e.g., easy availability of a large number of eggs, rapid and external development, and short generation time. Recently, the generation of hundreds of random zebrafish mutants has further enhanced its position as a vertebrate model in developmental analysis (Driever *et al.*, 1996; Haffter *et al.*, 1996). The zebrafish is particularly feasible for analysis of muscle-specific gene expression. The embryonic expression in skeletal muscle is easily observable, and plenty of muscle tissue is available from adult fish for molecular analysis. Transgenic studies can be carried out to analyze gene promoters (Gong *et al.*, 1991; Meng *et al.*, 1997). Moreover, expression of DNA injected directly into skeletal muscle has been demonstrated in the mouse (Wolff *et al.*, 1990; Vincent *et al.*, 1993) and could be adapted for the zebrafish.

In the present study, we isolated from the zebrafish a muscle-specific cDNA clone encoding an MLC2 protein. To develop a system for analyzing muscle-specific gene expression in zebrafish, we have further isolated its promoter and demonstrated by intramuscular injection of promoter–reporter gene constructs that it is active in muscle cells. Our study demonstrated the feasibility of characterizing muscle gene promoters in the zebrafish by direct injection of DNA constructs into skeletal muscle.

MATERIALS AND METHODS

cDNA cloning and sequencing

The full-length cDNA clone coding for zebrafish MLC2f was isolated by sequencing randomly selected cDNA clones from a cDNA library made from mixed stages of zebrafish embryos (Gong *et al.*, 1997). The 1.4-kb cDNA clone (E72) was sequenced completely by the dideoxynucleotide chain-termination method using the T7 Sequencing Kit (Pharmacia).

Northern blot hybridization

Total RNA was isolated from various tissues of adult fish and from embryos of different developmental stages using TRIzol reagent (GIBCO/BRL). The RNA (10 μg) was fractionated on 1.2% formaldehyde–agarose gels and transferred to GeneScreen membranes (DuPont-New England Nuclear) as previously described (Gong, 1992). The blots were prehybridized at 42°C in hybridization buffer (50% formamide, 5× Denhardt's solution, 4× SET [1× SET = 0.15 M NaCl; 1 mM EDTA; 20 mM Tris, pH 7.8], 0.2% NaPPi, 25 mM phosphate buffer, calf thymus DNA 250 μg/ml, and 0.5% SDS). Hybridization with a ^{32}P-labeled *MLC2f* cDNA probe was performed in the same hybridization buffer at 42°C overnight. Membranes were washed first with 2× SET/0.5% SDS and finally with 0.2× SET/0.1% SDS at 65°C and exposed to X-ray film for autoradiography.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization using a digoxigenin (DIG)-labeled riboprobe was carried out essentially as reported by Thisse *et al.* (1994). The *MLC2f* plasmid DNA was linearized by *Bam*HI, followed by *in vivo* transcription reactions with T7 RNA polymerase for the antisense RNA probe. Embryos of various stages were hybridized with the RNA probe in 50% formamide, 5× SSC, heparin 50 μg/ml, tRNA 500 μg/ml, and 0.1% Tween 20 at 70°C, followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and by staining with substrates nitroblue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indolil phosphate (BCIP) to produce purple insoluble precipitates. Some of the stained embryos were embedded in 1.5% agar-sucrose and sectioned on a cryostat (15 μm).

Isolation of zebrafish genomic DNA

Genomic DNA was isolated from a single individual fish by a standard method (Sambrook *et al.*, 1989). Briefly, an adult fish was quickly frozen in liquid nitrogen and ground into powder in liquid nitrogen. The ground tissue was then transferred to the extraction buffer (10 mM Tris, pH 8; 0.1 M EDTA, RNase A μg/ml, and 0.5% SDS) and incubated at 37°C for 1 h. Proteinase K was added to a final concentration of 100 μg/ml and gently mixed until the mixture appeared viscous, followed by incubation at 50°C for 3 h with periodical swirling. The genomic DNA was gently extracted three times by phenol equilibrated with Tris HCl (pH 8), precipitated by adding 0.1 volume of 3 M NaOAc and 2.5 volume of ethanol, and collected by swirling a glass rod, followed by a 70% ethanol rinse.

Isolation of MCL2f *gene promoter*

The *MLC2f* gene promoter regions were isolated by a linker-mediated polymerase chain reaction (PCR) method (Liao *et al.*, 1997). Briefly, zebrafish genomic DNA was digested respectively by *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Sac*I and modified by T4 DNA polymerase to generate blunt ends if the digested DNA had sticky ends. The digested genomic DNA was then ligated with a short linker DNA. Nested PCR was performed by two linker-specific primers, L1 (5′-GTTCATCTTTACAAGCTAGCG) and L2 (5′-TCCTGAACAATGCTGTGGAC), and two gene-specific primers, M1 (5′-CCATGT-CGAGACGGTATGTGTGA) and M2 (5′-GTGTGAAGTCTAAGAAGATCAAG), which are complementary to the 5′ end of the *MLC2* cDNA. The PCR products were gel purified and cloned into pT7 Blue vector (Novagen).

Promoter–reporter gene constructs

Three *MLC2f* promoter/CAT reporter gene constructs were made by insertion of a 1-kb, 2-kb, on 3-kb *MLC2f* promoter re-

gion into the *Hin*dIII/*Bam*HI sites of pBLCAT3 (Luckow and Schutz, 1987). The resulting constructs were named pMLC2f1/CAT, pMLC2f2/CAT, and pMLC2f3/CAT, respectively. A pituitary-specific promoter/CAT construct, −2.4sPRL/CAT, which contains 2.4 kb of promoter region from a prolactin gene of chinook salmon, was described previously (Elsholtz *et al.*, 1992). 5′ Unidirectional deletion clones were constructed from pMLC2f1/CAT (also named pMLC2f-1044CAT to be consistent with the nomenclature of the deletion constructs; see below) plasmid using the double-stranded Nested Deletion Kit (Pharmacia), and five of the deletion constructs were selected for promoter analysis; they are pMLC2f-1005CAT, pMLC2f-934CAT, pMLC2f-530CAT, pMLC2f-331CAT, and pMLC2f-79CAT, corresponding to −1005 bp, −934 bp, −530 bp, −331 bp, and −79 bp of the proximal promoter region, respectively. To generate an internal deletion in the promoter region, two outward PCR primers (out1: 5′-GGATCCAAGGGGCCTTC-GTCAGTAT; and out2; 5′-CCGGATCCCAACCTTAAGT-GAGG) were designed on the basis of the sequences adjacent to the deletion site (see Fig. 4 below), and outward PCR was carried out with two of the 5′ deletion constructs, pMLC2f-1005CA T and pMLC2f-79CAT, as templates. The outward PCR products were blunt-ended by T4 DNA polymerase and circularized by ligation, followed by a second PCR to amplify the deleted promoter region using two vector primers (CAT: 5′-AGCTTCCTTAGCTCCTG; and M13: 5′-GTAAAACGA-CGGCCAGT) and religation of the second PCR products into pBLCAT3.

Intramuscular injection of DNA

Zebrafish were purchased from a local aquarium store. Prior to injection, the fishes were anesthetized with 3-aminobenzoic acid ethyl ester (MS222; 100 mg/L) for a few minutes. A microliter syringe (Hamilton 26-gauge needle) was used to deliver plasmid DNA in phosphate buffered saline (PBS) into one flank of the fish. The site of injection was the skeletal muscle immediately rostroventral to the dorsal fin. Injection of an equivalent volume of PBS was used as a negative control in these experiments.

Chloramphenicol acetyltransferase assays

The rapid CAT assay method developed by Neumann *et al.* (1987) and Eastman (1987) was adopted. At the indicated time, injected fishes were sacrificed, and the muscle tissue around the injection site (about 50 mg) was excised and homogenized in 300 μl of 0.25 M Tris HCl (pH 7.8), followed by three cycles of freeze–thawing. The samples were then centrifuged at 14,000 rpm for 10 min. For CAT assay, 100 μl of the supernatant fluid was removed and heated at 65°C for 10 min to inactivate any endogenous CAT activity. The reaction mix (40 μl), consisting of 0.5 μl of 200 mM chloramphenicol (Sigma), 0.5 μl of ^{3}H-acetyl CoA (Amersham; 216 μCi/mM), 19.5 μl of 75 mM HCl, and 19.5 μl of 0.25 M Tris HCl (pH 7.8), was added. The samples were incubated at 37°C for 3 h, and 1 ml of 5 M urea was added to stop the reaction. The mixture was transferred to a scintillation vial containing 5 ml of nonaqueous scintillation fluid (BCS-NA; Amersham), and the samples were then counted in a scintillation counter.

β-Galactosidase staining

For β-galactosidase staining, the injected fishes were fixed by injection of a solution of 2% formaldehyde, 2 mM $MgCl_2$, 1.25 mM EGTA, and 0.1 mM PIPES (pH 7.0) into the muscle tissue around the DNA injection area and the peritoneum, followed by immersion in the fixative solution for 6 h at 4°C. The fishes were then incubated in permeabilization solution (PBS supplemented with 0.07 M NaCl, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) and stained for β-galactosidase activity by immersing them in the permeabilization solution containing 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ at 37°C in the dark for 2 h. Following incubation, the fishes were rinsed in PBS, and the muscle tissue around the injection site was excised and mounted in a 20% sucrose/1.5% agarose block, which was then equilibrated in 30% sucrose overnight. Finally, the embedded muscle tissue was sectioned with a cryostat microtome in both transverse and longitudinal orientation.

RESULTS

Isolation and characterization of zebrafish MLC2f *cDNA clone*

One of our expressed sequence tag (EST) clones from a zebrafish embryonic cDNA library, ZF-E72, shared significant homology with the fast skeletal muscle *MLC2f* cDNAs from other vertebrates and appeared to contain the full coding region (Gong *et al.*, 1997). The ZF-E72 clone was sequenced completely, and the cDNA insert is 1386 nt long, comprising an open reading frame of 169 amino acids. Another EST clone, ZF-A113, which is identical to ZF-E72 in the overlapping region, is 6 nt longer at the 5′ untranslated region (UTR). The combined zebrafish *MLC2f* cDNA sequence of 1392 nt is shown in Figure 1. The deduced amino acid sequence is highly homologous to that of other vertebrate fast skeletal muscle MLC2f proteins (>80% identity) and shares relatively low identities (<60%) with other MLC2 proteins. Thus, it is likely that the zebrafish *MLC2f* clone we isolated represents an ortholog of vertebrate fast skeletal muscle *MLC2f* genes. Because of the clear orthology, it is likely that the emergence of different MLC2 isoforms occurred before the divergence of fish and tetrapods. The amino acid sequence alignment among various vertebrate MLC2f proteins is presented in Figure 2.

It is worth nothing from Figure 1 that there are two potential polyadenylation sites, AATAAA, at nucleotides 797 and 1351. The presence of two populations of *MLC2f* mRNAs (~1.5 kb and ~1.0 kb) was confirmed by Northern blot hybridization (Fig. 3).

Skeletal muscle-specific expression of the zebrafish MLC2f *gene*

To examine expression of the zebrafish *MLC2f* gene, Northern blot hybridization was carried out. As shown in Figure 3A, *MLC2f* mRNA started to appear in embryos before 20 hpf, increased in the next 2 days, and remained high in the adult stage. To examine the tissue distribution of *MLC2f* mRNA, total RNAs were prepared from several adult tissues, including brain,

```
   1                                                    CTCTTCTTGATCTTCTTAGACTTCACACATACCGTCTCGAC   41
  42  ATG GCA CCC AAG AAG GCC AAG AGG AGG GCA GCA GGA GAG GGT TCC TCC AAC   95
   1   M   A   P   K   K   A   K   R   R   A   A   G   G   E   G   S   S   N   18

  96  GTC TTC TCC ATG TTT GAG CAG AGC CAG ATT CAG GAG TAC AAA GAG GCT TTC ACA  149
  19   V   F   S   M   F   E   Q   S   Q   I   Q   E   Y   K   E   A   F   T   36

 150  ATC ATT GAC CAG AAC AGA GAC GGT ATC ATC AGC AAA GAC GAC CTT AGG GAC GTG  203
  37   I   I   D   Q   N   R   D   G   I   I   S   K   D   D   L   R   D   V   54

 204  TTG GCC TCA ATG GGC CAG CTG AAT GTG AAG AAT GAG GAG CTG GAG GCC ATG ATC  257
  55   L   A   S   M   G   Q   L   N   V   K   N   E   E   L   E   A   M   I   72

 258  AAG GAA GCC AGC GGC CCA ATC AAC TTC ACC GTT TTC CTC ACC ATG TTC GGA GAG  311
  73   K   E   A   S   G   P   I   N   F   T   V   F   L   T   M   F   G   E   90

 312  AAG TTG AAG GGT GCT GAC CCC GAA GAC GTC ATC GTG TCT GCC TTC AAG GTG CTG  365
  91   K   L   K   G   A   D   P   E   D   V   I   V   S   A   F   K   V   L  108

 366  GAC CCT GAG GGC ACT GGA TCC ATC AAG AAG GAA TTC CTT GAG GAG CTT TTG ACC  419
 109   D   P   E   G   T   G   S   I   K   K   E   F   L   E   E   L   L   T  126

 420  ACT CAG TGC GAC AGG TTC ACC GCA GAG GAG ATG AAG AAT CTG TGG GCC GCC TTC  473
 127   T   Q   C   D   R   F   T   A   E   E   M   K   N   L   W   A   A   F  144

 474  CCC CCA GAT GTG GCT GGC AAT GTT GAC TAC AAG AAC ATC TGC TAC GTC ATC ACA  527
 145   P   P   D   V   A   G   N   V   D   Y   K   N   I   C   Y   V   I   T  162

 528  CAC GGA GAG GAG AAG GAG GAG TAA ACAACCTTGGAATCAAGAAAACGAAGAGAAGAACATGCA  590
 163   H   G   E   E   K   E   E   *                                          169

 591  TCCTCACAGCTTAATCTCCAGTCTGTTGTCTGGCCTTCTCTAACTTTTGTTTTCCTTCCTCCCTTTCTTG  661
 662  CTTTCTACCATCGTTGTTACTCCAAGCACTTACACTCTCCATCTTACCAAAGACTTGTCTCGCTGGGACTG  732
 733  AATTGGGAGGGTGGAGAGGAACACGACCACAGTGTCTGTCGAGTGGGGACATGGGATTGTTTCAATAAAA  803
 804  TGAACATCATTTCTGTATCTCTCACATTCTCTCTTTCTCTCTGTTTCTCACTCATTACCCACAACCCCTCT  874
 875  CTTTCATTTCAGTCAAGCTTGCATGTAAGTCGCTGCTTCTCTGCTGCAGTCTTAGGAGTTCAAACGAAGG  945
 946  CATCTATAGTTTGGGGCTGAAACATCTCTCTAGATCAATGTGGAAGAGTGCTCACTCTGAGGGGGAAAGAA 1016
1017  GCACGATGGAGTGATCTCACTCTATAATACAGGAACCAGTCATCATTCTCATTTCCTCCTCTGGTGGTTGA 1087
1088  CTAAAAAGAGAAAGAGAAAATGAGGGTTTTGTGCTGAGTGAGTTTAGCCTCCTAAAAGCGATGCCGAGCTC 1158
1159  ATCACAGAGGGAGTGAGAGGGACAGACCATCCTAGGAAGACAGCAGACAGGGACTGAAAGAAAACATAAC 1229
1230  CTCTTCACTCCCCCCTCTCCCCTCCTCTTCTCTATTTCTCTGTCCATCTTTTCTTTTTCTTTTTCTTTTT 1300
1301  TGCTTTCTGCATCTGGGCCTGCTTTGCTCTGCCAAACCTCTCCTGTAACCAATAAAAGACACAAACTGTG 1371
1372  AATAAAAAAAAAAAAAAAAA                                                    1392
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FIG. 1. The complete sequence of *MLC2f* cDNA clones and its deduced amino acid sequence. The numbers of nucleotide and amino acid residues are indicated on both sides. The two potential polyadenylation sites, AATAAA, are underlined.

eyes, gills, intestine, liver, skeletal muscle, ovary, and skin. The *MLC2f* mRNA was detected only in skeletal muscle.

To investigate the detailed pattern of *MLC2f* mRNA expression in developing embryos, whole-mount *in situ* hybridization was carried out with embryos of various developmental stages. The *MLC2f* mRNA first appeared faintly in the first few anterior somites in the ~14-somite stage (~16 hpf), which is earlier than the initial stage at which this RNA was detected by Northern blot analysis because the *in situ* hybridization approach is more sensitive (data not shown). During subsequent development, the *MLC2f* mRNA was detected exclusively in somites (Color Plate 1, panel A). To determine which region of the somite expresses *MLC2f* mRNA, the stained embryos were sectioned, and the signal was found exclusively in the fast skeletal muscle (Color Plate 1, panel B). No *MLC2f* mRNA was detected in the surface slow muscle, which can be defined by staining with an antibody against a slow muscle myosin (Devoto *et al.*, 1996) and by *in situ* hybridization with a slow myosin-binding protein C cDNA probe (our unpublished observation). At 48 hpf, *MLC2f* mRNA was also detected in fin buds and several pairs of eye and jaw muscles, which are also striated fast muscles. However, no *MLC2f* mRNA signal was ever detected in the heart. These observations indicate that the zebrafish *MLC2f* mRNA is specifically expressed in fast skeletal muscle but not in other types of muscles such as slow skeletal, cardiac, and smooth muscle.

Isolation of zebrafish MLC2 *gene promoter*

In order to further analyze the skeletal muscle-specific expression in the zebrafish, the *MLC2f* gene promoter was isolated by a linker-mediated PCR approach (Liao *et al.*, 1997),

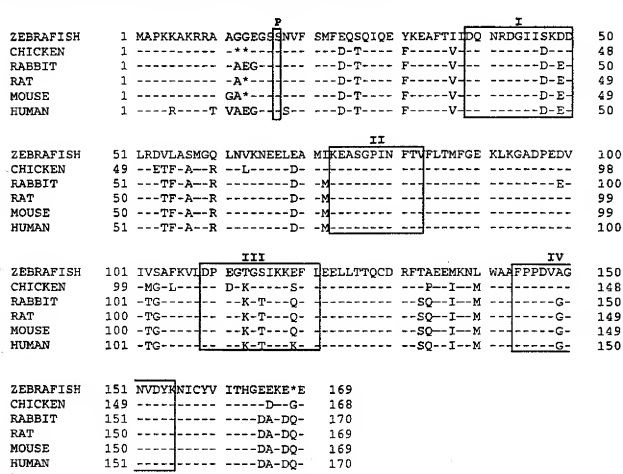


FIG. 2. Multiple sequence alignment of MLC2f proteins from various vertebrate species. The complete zebrafish sequence is shown. For other sequences, dashes indicate identical amino acid residues, while asterisks represent insertions of gaps for maximal alignment. The boxed regions (I–IV) represent the four conserved calcium binding domains of MLC2f. P indicates the site of phosphorylation of a serine residue that may be involved in the regulation of MLC2f activity (Michnoff *et al.*, 1986).

as briefly described in Materials and Methods. After two rounds of nested PCR, single DNA fragments of about 1 kb, 2 kb, and 3 kb were amplified respectively from *Eco*RV − , *Eco*RI-, and *Pst*I-digested, linker-ligated genomic DNAs. The 1-kb, 2-kb, and 3-kb fragments were then inserted into pT7 Blue vector. Sequencing was performed from both ends, and all three fragments had identical 3′ ends, indicating that they were amplified from the same gene. The sequence immediately upstream of the gene-specific primer M2 was identical to the 5′ UTR of the *MLC2f* cDNA clone; thus, the amplified DNA fragments were indeed derived from the *MLC2f* gene. The 1-kb fragment was sequenced completely, and the result is shown in Figure 4. A perfect TATA box was found 30 nt upstream of the transcription start site, which was defined by a primer extension experiment (data not shown).

For muscle gene expression, thee are two sets of well-characterized *cis*-elements: MEF2-binding site and E-box, the binding site for myogenic basic helix–loop-helix (bHLH) transcription factors such as MyoD and myogenin. Both elements can be found in the 1-kb promoter region. As indicated in Figure 4, there are four perfect E-boxes (CANNTG), one perfect MEF2-binding site, and two potential MEF2-binding sites, which share 90% identity with the consensus, YTA(A/T)$_4$TAR (Schwarz *et al.*, 1993; Olson *et al.*, 1995).

Zebrafish skeletal muscle is capable of expressing exogenously introduced DNA

As there is no zebrafish muscle cell line available, direct injection of DNA constructs into skeletal muscle becomes a method of choice for promoter analysis. To investigate the feasibility of the approach to study promoter activity, several preliminary experiments were carried out. First, the time course of CAT activity was examined after injection of a positive CAT DNA construct, **pBLCAT2**, which contains a strong and ubiquitous promoter from the herpes simplex virus thymidine kinase (*tk*) gene (Luckow and Schutz, 1987). The CAT activity was assayed 2 to 7 days after injection. As shown in Figure 5A, the activity increased rapidly from day 2 to day 4 and reached a plateau at day 5. Therefore, all subsequent CAT assays were performed 5 days after injection. Second, the optimal amount of DNA for injection was determined. Different amounts of pBLCAT2, ranging from 0.25 μg to 4 μg per fish, were injected. As shown in **Figure 5B**, there was a constantly high level

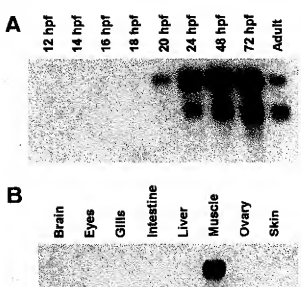


FIG. 3. Expression of zebrafish *MLC2f* mRNA revealed by Northern hybridization in developing embryos **(A)** and different adult tissues **(B).** The stages of embryos and adult tissues are indicated at the top of each lane. Adult RNA was prepared from whole fish.

of CAT activity when 1 to 4 μg of DNA was injected. Therefore, in this study, we injected 2 μg of DNA, which is about 3.4×10^{11} molecules for a 5.3-kb plasmid (pMLC2f1), per fish for promoter analysis.

To confirm the transformation of muscle cells by direct DNA injection, a *lac*Z DNA construct, pCMVβ (Clontech), which contains a strong cytomegalovirus promoter and a β-*gal* gene, was injected. The injected fish were fixed 5 days after injection and stained for β-galactosidase activity. Positive staining was observed within the striated muscle cells in the injection area. Thus, a single injection can transform several muscle fibers (Color Plate 2, panels B and C), indicating that, indeed, the plasmid DNA injected was taken up by the myofibers and the *lac*Z gene had been transcribed and translated. In comparison, injection of PBS buffer produced no β-galactosidase activity (Color Plate 2, panel A).

Functional analysis of MLC2f *promoter by direct injection into skeletal muscle*

In order to test the function of the isolated *MLC2f* promoter regions, the 1-kb, 2-kb, and 3-kb promoter fragments were inserted into a CAT reporter gene vector, pBLCAT3 (Luckow and Schutz, 1987). The resulting gene constructs, pMLC2f1/CAT, pMLC2f2/CAT, and pMLC2f3/CAT, were injected into zebrafish skeletal muscle. As shown in Figure 6A, all of the three promoter constructs produced high levels of CAT activity, which were higher than that generated by the viral *tk* promoter construct, pBLCAT2. In contrast, the *prolactin* promoter had minimal activity in the muscle cells. These results indicate that the *MLC2f* promoter is indeed functional in muscle.

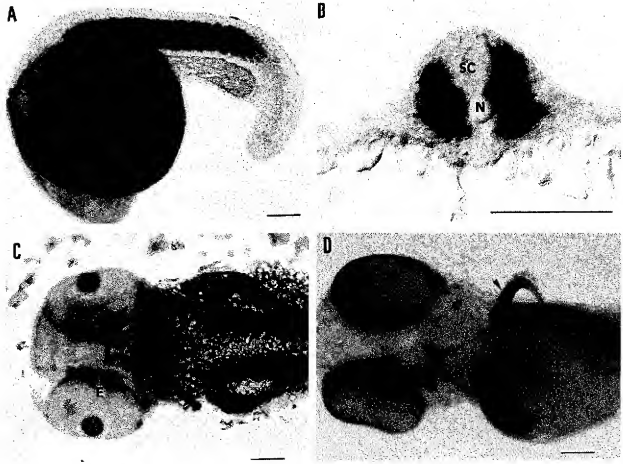
As shown in Figure 6A, there was no apparent difference in CAT activity among the three MLC2f/CAT constructs having the *MLC2f* promoter regions from 1 kb to 3 kb; thus, the *cis* elements important for muscle gene expression may be located within the 1-kb proximal region. To map the *cis* elements important for muscle gene transcription, unidirectional deletion from the 5′ end of the 1-kb (or 1044-bp) CAT construct, pMLC2f1/CAT or pMLC2f-1044CAT), produced various lengths of the promoter fragment. Five constructs, each carrying a promoter of a different length (1005, 934, 530, 331, and 79 bp), were selected for muscle injection and CAT assay. As shown in Figure 6B, there was essentially no difference in promoter activity among the various deletion constructs. The deletion up to -79 retained strong activity, comparable to that of the unmodified 1-kb promoter. This result indicates that -79 bp is sufficient to support strong expression in muscle cells.

In the 79 bp of the promoter region, the only obvious DNA element, in addition to the TATA box, is an MEF2-binding site located at -56 to -46. Thus, this MEF2-binding site may be an important muscle-specific *cis* element for *MLC2f* gene expression. To confirm this hypothesis, we made an internal deletion to remove the MEF2-binding site from both pMLC2f-79CAT and pMLC2f-1005CAT by an outward PCR approach. The two internal-deletion constructs were also used for muscle injection and CAT assay, together with the undeleted ones for comparison. Internal deletion of the MEF2-binding site from the 79-bp fragment caused a dramatic decrease of CAT activity, 80% below that of the undeleted one, indicating the essential function of the MEF2-binding site for *MLC2f* gene expression in muscle (Fig. 6C). However, deletion of the same MEF2-binding site from pMLC2f-1005CAT was followed by retention of a high level of promoter activity. This phenomenon may be explained by the presence of additional MEF2-binding sites in the -1005-bp region, and these additional upstream MEF2 elements may compensate for the loss of the proximal MEF2 site.

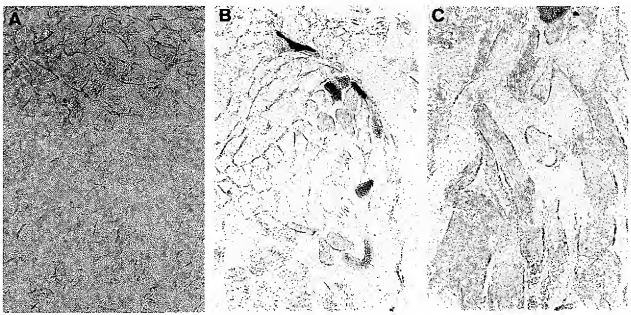
DISCUSSION

Expression of fast skeletal muscle MLC2f *gene in zebrafish*

In the present study, a zebrafish *MLC2f* cDNA clone has been isolated and characterized. The deduced protein sequence is highly homologous to those of vertebrate MLC2f, the fast skeletal muscle isoform. The zebrafish *MLC2f* gene is expressed in both embryos and adult fish, and the expression is specifically in fast skeletal muscle. The onset of *MLC2f* expression is much later than those of the myogenic basic bHLH genes such as *myoD* and *myogenin* (Weinberg *et al.*, 1996). From the previous study by Weinberg *et al.* (1996), zebrafish *myoD* transcript is first detected at 7–7.5 hpf, when the somites are not overtly formed. The *myogenin* transcript is first detected at 10.5 hpf, shortly after the first somite is formed. In contrast, no *MLC2f* mRNA can be detected until 16 hpf. At this stage, about 14 somites have been formed, and weak *MLC2f* expression can be detected in the first few anterior somites but not in the newly



Color Plate 1. Expression of zebrafish *MLC2f* mRNA revealed by whole-mount *in situ* hybridization. Embryos at selected stages were hybridized with the *MLC2f* antisense riboprobe. **A.** Side view of a 22-hpf embryo. **B.** Transverse section through trunk of a stained embryo (24 hpf). **C.** Dorsal view of the rostral part of embryo (48 hpf) shows *MLC2f* mRNA in eye and jaw muscles. **D.** Ventral view of the rostral part of an embryo (48 hpf) shows *MLC2f* mRNA expression in fin bud, as indicated by an arrowhead. All bars represent 100 μm. Abbreviations: E, eye; N, notochord; J, jaw; and SC, spinal cord.



Color Plate 2. The β-galactosidase activity in DNA-injected skeletal muscle. **A.** Cross-section of the muscle injected with PBS. (Original magnification 265×). **B.** Cross-section of muscle injected with pCMVβ. (Original magnification 265×). **C.** Longitudinal section of muscle tissue injected with pCMVβ. (Original magnification 530×). Note the striations in the fast muscle fibers.

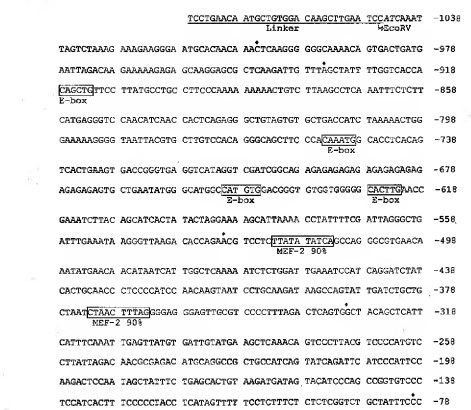


FIG. 4. The complete sequence of the proximal 1044-bp promoter region of the *MLC2f* gene. Two types of muscle *cis* elements, E-box (CANNTG), and MEF2-binding site [(C/T)TA(T/A)$_4$TA(A/G)], are shown in boxes. The TATA box and transcription start site +1 are shown in boldface. Both the 5′ linker DNA sequence and the downstream M2 primer are indicated, and the remaining half *Eco*RV site, ATC, immediately following the linker DNA is shown in italics. Diamonds indicate the first nucleotide of each of the 5′ unidirectional deletion constructs. The sequences of the two outward PCR primers, out1 and out2, are also indicated, and the extra sequences for *Bam*HI sites are shown in bold.

formed posterior somites. In the late stages of embryos, *myoD* is also expressed in the fin bud and eye and jaw muscles (Weinberg *et al.*, 1996). Similarly, *MLC2f* mRNA can also be detected in these cells. Therefore, the *MLC2f* gene is likely a direct regulatory target of myogenic bHLH transcription factors such as MyoD and myogenin. Consistent with this view, the isolated *MLC2f* promoter contains several E-box sequences (CANNTG), which are the binding sites for myogenic bHLH factors, in the 1 kb of the proximal promoter region. Because *MLC2f* is expressed only in well-formed somites when muscle cells are differentiated, it can be used as a differentiation marker for fast skeletal muscle.

MLC2 *promoter and MEF2-binding site*

Thus far, muscle gene promoters have been characterized only in several species of higher vertebrates (Henderson *et al.*, 1989; Horlick and Benfield, 1989; Gustafson and Kedes, 1989; Kuisk *et al.*, 1996), but not in fish. Generally, the *cis* elements for muscle-specific transcription are located within a few hundred basepairs of the proximal promoter region (Arnold *et al.*, 1988; Lee *et al.*, 1994; Catala *et al.*, 1995). Some muscle-specific enhancers can be found further upstream beyond 1 kb (Horlick and Benfield, 1989; Asakura *et al.*, 1993). In the present study, by a series of deletion analyses, we found that a 79-bp proximal promoter region from the zebrafish *MLC2f* gene is sufficient to support a high level of expression in muscle cells. This observation may not be surprising, as previously, Braun *et al.* (1989) reported that 69 bp of 5′ flanking region from the chicken cardiac *MLC2A* gene is sufficient to allow muscle-specific transcription. In our study, the −79-bp promoter produced the same level of CAT activity as a 3-kb promoter, suggesting that there is no other major enhancer sequence within the 3-kb region upstream of the RNA start site. However, it cannot be ruled out that some enhancers are located beyond the 3-kb region or in a downstream intron.

Further analysis of the 79 bp of the promoter region indicated the presence of a perfect MEF2-binding site. Deletion of this site from the 79-bp promoter caused a dramatic decrease of promoter activity in muscle cells, indicating the important role of the MEF2-binding site in maintaining *MLC2f* gene expression. Although the deleted MEF2 site appears to be essen-

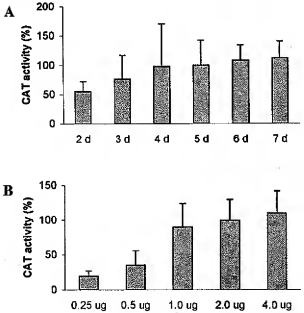


FIG. 5. The CAT activity in skeletal muscle after injection of various CAT constructs. **A.** Time course of pBLCAT2 expression. Fishes were injected with 2 μg of pBLCAT2, and CAT assays were carried out at indicated days (≥ 5 at each timepoint). The CAT activity at 5 days postinjection is set arbitrarily to 100%. **B.** Dosage-dependent expression of pBLCAT2. Fishes were injected with various amounts of pBLCAT2, and CAT assays were carried out 5 days postinjection (N ≥ 5 for each assay). The CAT activity in fishes injected with 2 μg of DNA is set arbitrarily to 100%. In both panels, the standard errors are indicated by T bars.

tial to activate the *MLC2f* gene in muscle cells, it is unlikely to be the only *cis* element for muscle specific transcription. Consistent with this idea, deletion of the same MEF2-binding site from a 1-kb promoter construct resulted in no apparent loss of promoter activity, indicating the presence of other functional *cis* element(s) for transcription in muscle cells. It is worth noting that there are two other potential MEF2 sites present in the 1-kb upstream region, and these upstream sites may compensate for the effect of deletion of the proximal MEF2 site. Acquisition of more than one muscle-specific element in the same gene promoter has obvious advantages; for example, any spontaneous mutation of one of these *cis* elements will not have a detrimental effect.

MEF2 is capable of binding to an A/T-rich sequence required for the activation of many cardiac and skeletal muscle-specific promoters/enhancers and is a key regulator for both the cardiac and skeletal muscle lineages (Gossett *et al.*, 1989; Cserjesi and Olson, 1991). It is also a regulator of the myogenic bHLH genes (Edmondson *et al.*, 1992). Several zebrafish MEF2 genes have been recently cloned, and they are expressed in the early stages of somitogenesis and myocardial cell differentiation (Ticho *et al.*, 1996). Usually, MEF2 functions in conjunction with myogenic bHLH factors (for reviews, see Firulli and Olson, 1997; Shore and Sharrocks, 1995). Therefore, it is frequently noted that some E-box sequences are found near the MEF2-binding site (Schwarz *et al.*, 1993). However, in the present study, it seemed that a single MEF2 site was sufficient to activate the *MLC2f* promoter in muscle cells. Although there are two imperfect E-boxes, −71 CACTTA and −46 CAAGGG, in the 79 bp of proximal promoter region, whether these imperfect E-boxes play any role in muscle-specific transcription remains unknown.

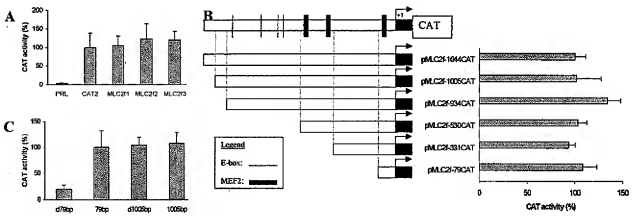


FIG. 6. Functional analysis of *MLC2f* promoters by direct injection into skeletal muscle. **A.** Expression of *MLC2f* promoter/CAT constructs. Fishes were injected with −2.4sPRL/CAT, pBLCAT2, pMLC2f1/CAT, pMLC2f2/CAT, and pMLC2f3/CAT, which have been simplified as PRL, CAT2, MLC2f1, MLC2f2, and MLC2f3, respectively, in the figure. The CAT activity of pBLCAT2 is set arbitrarily to 100%. **B.** Mapping of muscle *cis* elements in *MLC2f* promoter. Fishes were injected with successively 5′-truncated *MLC2f* promoter/CAT constructs. The two important muscle-specific elements, E-box and MEF2-binding site, are indicated as thin and thick bars, respectively. The CAT activity of the undeleted construct (pMLC2f-1044CAT) is set arbitrarily to 100%. **C.** Test of internal deletion of the proximal MEF2-binding site. Fishes were injected with pMLC79ΔMEF2-CAT (MEF2 deleted), pMLC79-CAT, pMLC1005ΔMEF2-CAT (MEF2 deleted), and pMLC1005-CAT, which have been simplified as d79bp, 79bp, d1005bp, and 1005bp, respectively, in the figure. The CAT activity of pMLC2f-79CAT (79bp) is set arbitrarily to 100%. For each CAT assay, at least eight fishes were used for injection of the same DNA construct. In all cases, standard errors are indicated by T bars.

A valid promoter analysis approach by direct injection of DNA into skeletal muscle

There are two basic approaches using promoter/reporter gene constructs to analyze promoter activity. One is to transfect DNA constructs *in vitro* into cultured cells, and the other is a transgenic approach in which DNA is introduced into fertilized eggs. In zebrafish, very few cell lines are available, and there is no muscle cell line. The transgenic approach is tedious and sometimes unreliable in fish because of mosaic segregation of the transgene in early embryos (for review, see Gong and Hew, 1995). Therefore, direct injection of DNA into skeletal muscle is a convenient approach to characterize muscle-gene promoters. Our work demonstrated the feasibility and validity of this approach, as evident from the fact that the promoter from the *MLC2f* gene produced a high level of reporter gene activity, whereas a non-muscle-gene promoter was basically inactive (see Fig. 6A). By deletion analyses, we have identified a single MEF2-binding site which is essential for *MLC2f* gene transcription in muscle cells.

Compared with the *in vitro* system, direct injection into muscle has several advantages. First, zebrafish are in large supply, whereas cultured zebrafish cells are not readily available. Second, the injected fish are easily and conveniently maintained compared with the stringent and aseptic conditions required for cell culture, where contamination could be a major problem. Third, injection of zebrafish gene promoter constructs into zebrafish muscle provides a homologous *in vivo* system to analyze muscle-specific genes, and the information obtained would be more reliable than that from the studies performed in a heterologous system. This work represents the first report of the use of the direct muscle injection approach to analyze muscle-specific gene promoter in fish.

ACKNOWLEDGMENTS

We thank Dr. Vladimir Korzh for his help in *in situ* hybridization analysis and critical comments on the manuscript and Mr. H.K. Yip for photograph reproduction. This work was supported by an NUS academic research grant. Y. Xu and J. Liao were supported by NUS postgraduate scholarships. The GenBank Accession Number for the cDNA sequence reported in this work is AF081462.

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Received for publication August 10, 1998; accepted in revised form September 22, 1998.

EXHIBIT 4

25/02

① Digestion with BamHI.

DNA fragment (7kbp, 100ng)	10 μl
10X Buffer	2 μl
10 X BSA	2 μl
BamHI	1 μl
H2O	5 μl
	/20μl 37°C incubation for 1 hr

② Add 180 μl TE, 200 μl phenol mix, 14000rpm X5'. precipitation DNA with ethanol -80°C for 2 hrs

③ 14000 rpm X3' at 4°C. wash with 70% ethanol, air dry.

④ dissolve in 20 μl H2O

⑤ Klenow modification.

DNA	16.5 μl
10×Klenow buffer	2 μl
Klenow	0.5 μl
dNTP	1 μl
	/20μl 37°C incubation for 30'.

⑥ Ligation.

modified DNA	10 μl
5× T4 ligase buffer	3 μl
T4 ligase	1 μl
	/15μl 16°C incubation overnight.

26./02.

① PCR check the effect of ligation

for 1 sample

10×PCR buffer	5
2.5mM dNTP	2
$MgCl_2$	3
CAT primer	0.5
M13 primer	0.5
Taq	0.2
Template	1
H_2O	37.2
	/50 ml

Template: ① 79bp ligation
② 1005bp ligation
③ 1005bp plasmid (no deletion)
④ 79bp plasmid (no deletion)

② purify the 79 bp and 1005bp fragment in 15 ml H_2O

digest with SpeI and SphI.

DNA fragment	15 ml
10× Buffer 2	2 ml
10 × BSA	2 ml
SpeI	0.5 ml
SphI	0.5 ml
	/20 μl

37°C incubation for 1 hr.

(The SpeI doesn't work! ?)

27/02

① PCR repeat

The 1005bp band is still very faint.

⑥ PCR again with undigested 79 bp and 1057bp fragments as negative control.

Template ① 79 bp ligation 1ml
② undigested 79 bp fragment 0.2ml
③ 1057bp ligation 1ml
④ undigested 1057bp fragment 0.2ml
⑤ 1057bp plasmid as positive control

touchdown PCR program
68°C → 59°C → 58°C → 57°C → 56°C (30 cycles)

cut the 79 bp band and half the 1057bp band

⑦ use the 1057bp ~~fragment~~ fragment (in the gel, freeze and thaw twice) as the template to repeat the PCR
it didn't work

28/02

PCR again using 1057bp fragment (as yesterday) as template to do second round PCR.
It doesn't work

2/3. (from maxi-prep)

① cut MLCP284-CAT and MLCP79 (M13-, M13 and CAT PCR product) fragment with HindIII and SpeI. (promega)

	MLCP284-CAT	M13-79bp fragment
~~MLCP284-CAT~~ DNA	2 ml	15 ml
Promega multi-core buffer	2 ml	2 ml
BSA (1mg/ml)	0.2 ml	0.2 ml
HindIII (10u/ml)	1 ml	0.5 ml
SpeI (10u/ml)	1 ml	0.5 ml

③. Run the digestion on the gel.

③. Gel purify the CAT vector (HⅢ/SpeⅠ) frag. into 30 ml H_2O
Gel purify the MEF2-79 frag (HⅢ/SpeⅠ) into 15 ml H_2O

④ Ligation

MEF2-79 (HⅢ/SpeⅠ)	15 ml
CAT vector (HⅢ/SpeⅠ)	0.5 ml
5X T4 ligase buffer	4 ml
T4 ligase	1 ml / 20 ml

4°C incubation overnight.

3/3.

①. The ligation was carried out at room temperature for 2 hrs more, then do transformation

4/3.

①. PCR screen the 79-CAT (HⅢ/SpeⅠ) ligation.

primers: M13 and CAT primer

~~use wrong primers. should use M13 and M2 primer~~

②. PCR again.
using M13 and M_2 primers.

5/3

① Inoculate 20 colonies

2. 6. 9. 11. 12. 13. 14. 16. 22. 23. 24. 28. 30. 31. 32. 40. 41. 42. 43. 45

② MEF2-lws (M13 & CAT PCR) fragment digestion with HindIII and SpeI, Ligate with CAT vector. (HindIII/SpeI cut)

6/3

① Sequencing reaction

A: 2. 6. 9. 11. 12

C: 13. 14. 16. 22. 23

G: 24. 28. 32. 41. 32. 40

T: 40. 41. 42. 43. 45

② Transform 7.5 ml MEF2-lws-CAT (HindIII/SpeI) ligation

7/3

① Run sequencing gel.

② PCR screening MEF2-lws-CAT (HindIII/SpeI) ligation plate using M13 and M2 primers.

A. 1/10 ligation plate #1-#24

PCR volume: 28 ml.

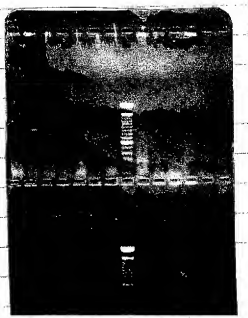
9600 machine

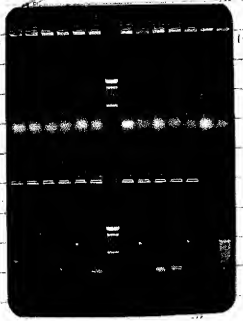
B. 9/10 ligation plate #1-#24

PCR volume: 28 ml

94°C 5' 9600 machine

94°C 30"





3/3.

Inoculation MEF2-IWS-CAT (HindIII/pst) ligation 7/10 plate colonies

#4 #12 #18 #20 #XL

7/3.

① Plasmid microprep in 25 μl H₂O

② Sequencing reaction. 79-12.

IWS-4, IWS-12, IWS-18, IWS-20, IWS-XL

10/3.

on sequencing gel

11/3.

Read sequence.

MEF-2

original sequence : CTTAAGGTTGGGTCTATAATTAGCAAGGGGCC

mutanted " : TTAAGGTTGGGATCGGATCCAAGGGGCC

MEF2R

CCCTAGGCC

MEF2A

?/3.

Transformation MZf2-79-CAT #12
MZf2-1005-CAT #20
MCKP5-CAT #19

43.41
CAT

16/3.

@ PCR check transformation.
for MZf2-79-CAT, MZf2-1005-CAT and ~~MZ~~ MLCP-79-CAT, use M13, M2.
for MCKP5-CAT use M13, MCK4.

① MCK5-CAT #1. ② MCK5-CAT #2
③ MCK5-CAT #3 ④ MZf2-79 #1
⑤ MZf2-79 #2. ⑥ MZf2-79 #3
⑦ MZf2-1005 #1 ⑧ MZf2-1005 #2
⑨ MZf2-1005 #3 ⑩ MLCP-79 #12
⑪ MLCP-79 #13 ⑫ MLCP-79 #14
⑬ MLCP-79 #15.

①②③ ④⑤⑥⑦⑧⑨ ⑩⑪⑫⑬

Inoculation MCK5-CAT #3.
MZf2-79-CAT #2
MZf2-1005-CAT #3
MLCP-79-CAT #13
} in 100 ml LB/Amp broth, 37°C incubating overnight.

13

17/3.

1. Plasmid maxi prep { MCK5-CAT, MZf2-1005-CAT. } in 80 ml TBS

2. check plasmid (maxi prep) by BamHI digestion
DNA 0.5 ml
① MCK5-CAT

① ② ③

3. Incubation. MEF2-7J-CAT and MLCP-7J-CAT again. in two ml LB/Amp. 37°C incubation overnight.

18/07

a. plasmid Maxi prep { MEF2-7J-CAT / MLCC-7J-CAT } > in 80 ml PBS

b. Check concentration.

		A_1	A_2	A_1/A_2	[DNA]
MLCP-7J-CAT	100 d	1.707	0.932	1.7928	
	200 d	0.867	0.490	1.7690	8.67 ng/ml
MEF2-7J-CAT	100 d	1.270	0.710	1.7887	
	200 d	0.632	0.359	1.7605	6.32 ng/ml
MEF2-10kb-CAT	100 d	1.225	0.682	1.7955	
	200 d	0.589	0.332	1.7726	5.89 ng/ml
MLCP-1.1kb-CAT	100 d	1.014	0.566	1.7922	
	200 d	0.492	0.279	1.7627	4.92 ng/ml

To make 1 ng/ml solution (50 ml in total)

MLCP-7J-CAT 5.77 ml + 44.23 ml PBS

MEF2-7J-CAT 7.91 ml + 42.09 ml PBS

MEF2-10kb-CAT 8.49 ml + 41.51 ml PBS

MLCP-1.1kb-CAT 10.16 ml + 39.84 ml PBS

19/07

Muscle injection

MEF2-7J 8 fish	MLCP-7J 8 fishes

26/07

CAT assay

	POS	CTIME	CPM1	SQPI
N	1		47.30	198.19
P	2		1532.40	139.34
Negative control	3		85.20	170.66
	4		75.90	161.13
MB2-79	5		1028.40	134.74
	6		218.80	125.96
	7		698.70	134.32
	8		197.50	134.10
	9		138.30	176.70
	10		226.70	129.25
	11		311.40	129.82
	12		190.80	124.49
MLG 79	13		527.30	131.87
	14		314.50	129.22
	15		166.90	126.87
	16		1577.20	136.65
	17		699.30	135.06
	18		1237.40	135.58
	19		94.10	156.57
	20		1083.60	136.86
MB2-105	21		1104.70	135.60
	22		772.70	136.90
	23		1199.30	134.08
	24		915.20	135.24
	25		1301.90	136.12
	26		1367.70	133.69
	27		148.90	120.52
	28		1295.60	134.15
	29		639.40	130.77
MLG 11	30		1088.60	133.80
	31		69.60	205.18
	32		112.50	157.88
	33		906.50	131.71
	34		907.60	132.69
	35		1552.00	136.53

N = 80.55

MB2 79 = 352.5

272

MLG 79 = 804.9

720.5

MB2-105 = 971.7

897.2

MLG 11 = 113.7

122.2

RAW DATA

191.7	377.8	196.4				
167.8	542.1					
187	304.2	364.6				
839.8	376.8	193.8	353.2	577.2		
711.8	555.6	288.4	367.7	653	491.7	427.9
562.2	725.7	601.7	212.6	497.6		

	$\bar{N}$	SR
2 d	265.3	81.66667
3 d	354.95	187.15
4 d	449.9333	341.7778
5 d	464.16	195.1672
6 d	499.4429	120.5918
7 d	519.96	131.888

35	72.7	140.8	
316.6	81	203.7	61.3
527.2	192.7	628.7	335.9
~~60~~			
642.1	603.1	293.7	

	$\bar{N}$	SR
0.25 mg	89.5	34.2
0.5 mg	165.65	94.5
1.0 mg	421.175	156.825
2.0 mg	465.6875	137.3075
4.0 mg	512.9667	146.1778

18.2	19.8	15.6	13.5		
644.8	317.1				
562.2	725.7	601.7	212.6	497.6	
387.9	1060.2	755.3	744.3	367.7	535
479.4	674.3	551.5	616.1	736.4	

PRL	17.16667	3.3
CAT 2	496.6	192.95
MLC 2.1	519.96	131.888
MLC 2 2	610.9	208.3143
MLC 2.3	595.3857	114.6409

(1) Time course

2 d.	255.3	49.1%
3 d	355.0	68.3%
4 d	451.9	86.9%
5 d	~~464.4~~ 464.2	89.3%
6 d	497.4	96.0%
7 d	520.0	100%

(2) Dose

0.25 μg	89.5	17.4%
0.5 μg	165.7	32.3%
1.0 μg	421.1	82.1%
2.0 μg	465.7	90.8%
4.0 μg	513.0	100%

(3) Different constructs

PRL	17.2	3.5%
CAT2	~~496~~ 496	100%
MLC 2-1kb	~~519~~ 520.0	104.7%
MLC 2-2kb	610.9	123.0%
MLC 2-3kb	595.4	119.9%
Ck		

(4) Deletion.

1045bp	536.9 ± 60.4	~~100%~~	100%
1005bp	546.2 ± 135.5	~~100%~~	101.7%
924bp	720.4 ± 71.5	~~134%~~	134.2%
~~808bp~~	~~389.5 ± 80.8~~	~~72%~~	72.5%

4	594.80	131.74	
5	172.60	126.26	
6	523.10	136.80	
7	134.30	121.29	
8	86.80	195.84 ×	
9	127.90	162.14	
10	203.30	122.47	
11	~~123.70~~	~~164.29~~	
12	353.50	132.62	
13	161.70	123.65 ×	
14	89.90	171.11	

Wallac 1400 DSA, P27AS069.DAT, 4/2/1998, page 1

P27AS069.DAT

POS	CTIME	CPM1	SQP1
15		705.10	132.87
16		370.60	132.32
17		637.40	134.16
18		77.50	179.88 ×
19		657.10	135.79
20		674.80	133.53
21		498.70	130.51
22		687.00	130.38
23		458.60	131.60
24		550.10	131.64
25		519.80	133.49
26		171.00	119.84 ×
27		552.50	132.32
28		279.60	129.60
29		516.80	132.86
30		42.60	206.02 ×
31		80.10	176.20 ×
32		564.50	133.03
33		450.40	134.40
34		776.60	137.98
35		65.50	177.19
36		263.00	130.95
37		379.60	133.34
38		264.60	129.66
39		66.00	192.28

[redacted]

EXHIBIT 5

In situ Hybridization of ARP probe

2/3/98 Also Transformation

3/3/98 Inoculate 2 colonies of A150

4/3/98 ① plasmid miniprep A150 ① A150 ②

② Single digestion by BamHI

plasmid	10 μl
10X buffer	2 μl
1% BSA	2 μl
BamHI	1 μl
H2O	5 μl

37°C incubation for 2 hrs

BamHI digestion

0.5 kb/lane

③ Size-fractionated on agarose gel.

④ phenol treat once. (Add TE to 200 μl, add phenol 200 μl mix 14000rpm x5' take the upper 200 μl liquid, add 20 μl NaOAc, pH5.2, 400 μl pure ethanol -80°C for 30m)

⑤ 14000rpm x30' at 4°C. wash with 70% ethanol 14000rpm x15' at 4°C air dry.

⑥ dissolve in 10 μl DEPC treated water, check concentration.

0.5 μl/lane

5/3/98 Making dig-RNA probe.

DNA (digested Ahd① plasmid)	4 μl
5X transcription buffer	4 μl
10 x dig-NTP mix	2 μl
RNAsin	1 μl
DTT (100mM)	1 μl
T7 RNA polymerase	1 μl
H_2O	7 μl
	20 μl

37°C incubation for 2hrs.

Add 2 μl DNAase I, 37°C incubation for 15 min.

Add 1 μl EDTA to stop the reaction.

Add 2.5 ml Cicla, 75 μl cold pure ethanol. -80°C put for 5 hrs.

14000rpm X 30', wash with 70% ethanol. air dry

Dissolve in 50 μl DEPC treated water.

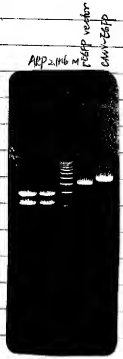
28/03/98 The APP-8kb-EGFP is not strong enough, so try use APP2.1kb-EGFP instead (with intron)

① Digestion

Plasmid (APP2.1kb in pT7 #32)	10 μl
10 x Buffer (BamH1)	2 μl
10 x BSA	2 μl
BamH1 (20u/μl)	1 μl
EcoR1 (60u/μl)	0.3 μl
H_2O	4.7 μl
	20 μl

37°C incubation.

check CMV-EGFP construct made by QIAGEN miniprep by BamH1 digestion (27/03/98)
(use empty pEGFP vector as control)



② Gel purify the 2.1kb APP fragment in 30 μl H_2O

③ Ligation

DNA	10.5 μl
5 x ligase buffer	3 μl
T4 ligase	1 μl
pEGFP (BglI/EcoR1)	0.5 μl
	15 μl

16°C incubation overnight.

ARP P0 Promoter / EcoRV digested fragment (2.1kb):

5' TCTGAACAATGCTGTGGACAAGCTTGAATTCATCTCTATTAAGAA
Linker DNA
ACACTTAAAATATATATGCGTTACGAATTAAAAACAAAACACGAT
CATTTTAATTTGTGTTGTATAATTTTACATTTTGTAAGTATTATTTT
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ATAAGATGCACACAAGGYAGGTGTAAAAGTATTGCTTGTGTTTGT
AATCCTCAGATTTTACAACCTTGTCTTTAAACCGGCTGTTCACCGA
ARP2: 3' ACAAGTGGCT

TCCTTGGAAGGGATCC 3'
AGGAACCTTCCCTAGG 5'

ARP P0 Promoter / PstI digested fragment (457 bp):

5' TCCTGAACAATGCTGTGGACAAGCTT
Linker DNA
GAATTCGGGACAATGGCCCAACAGGATTGGTGACCCCTGCCTCAAG
CCATCACAAATGCATTATGGTACTAAGAAATGTTGCAGGTTCAGTT
ATGGACAGGCTGTTGCAGTNTTGTTCGTCGGTNCCCCCTGCACAAA
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GTAATCCTCAGATTTTACAACCTTGTCTTTAAACCGGCTGTTCACCG
ARP2: 3' ACAAGTGGC

ATCCTTGGAAGGGATCC 3'
TAGGAACCTTCCCTAGG 5'

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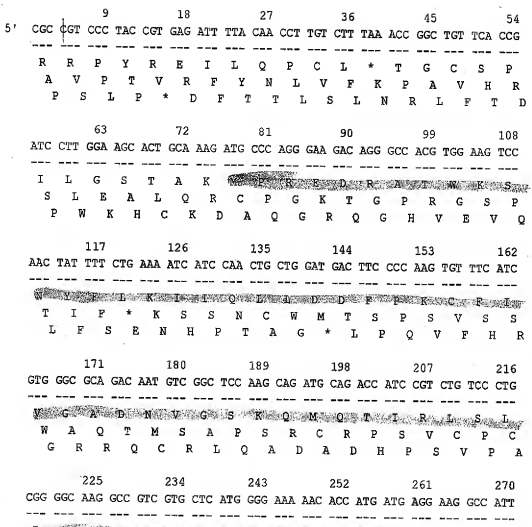
ARP2 primer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P S L P * D F T T L S L N R L F T D
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S L E A L Q R C P G K T G P R G S P
P W K H C K D A Q G R Q G H V E V Q
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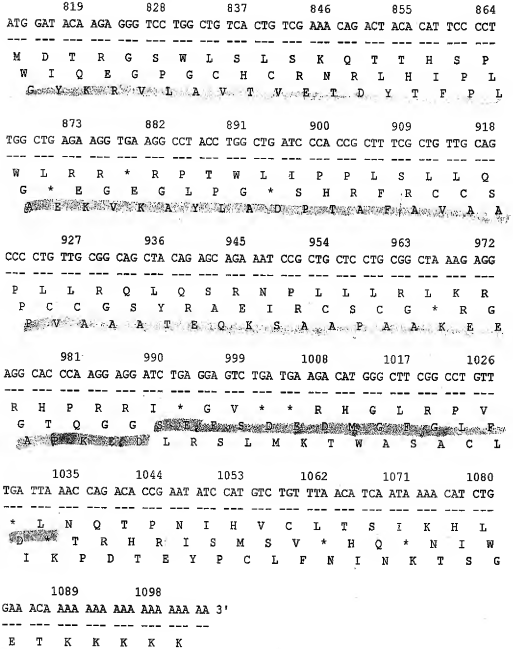
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Dyeset/Primer:	DT {BD Set Any-Primer}
Comb:	
Instrument Name:	377 # 97042603
Collect Vers.:	2.1

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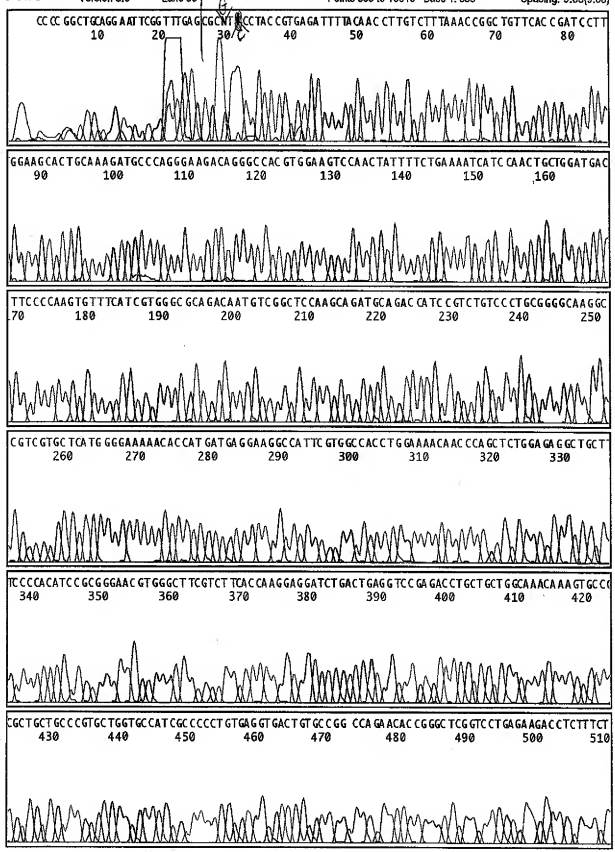
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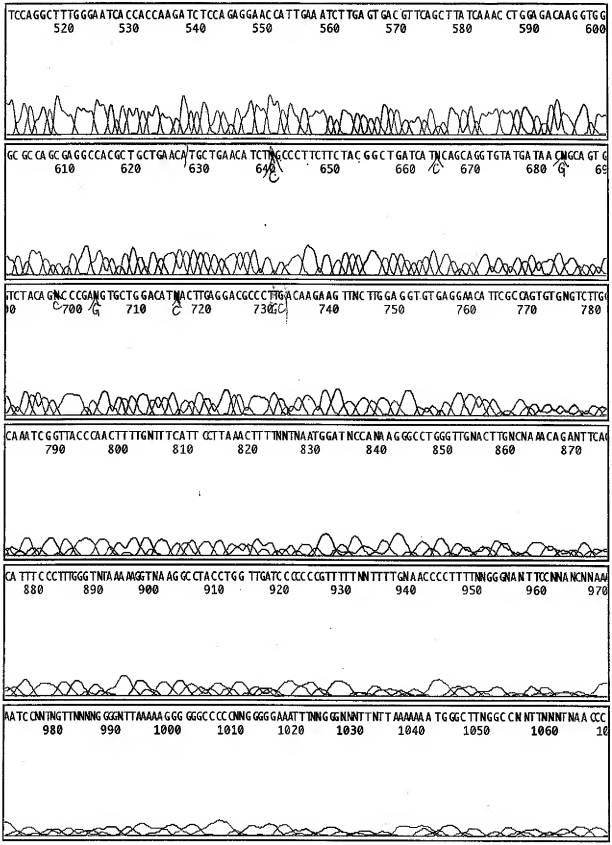
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No. of Channels:	194
Length:	1031
Run started at:	20/7/1998, 14:33
Run stopped at:	20/7/1998, 23:34
Gel:	Gel File
Dyeset/Primer:	DT {BD Set Any-Primer}
Comb:	
Instrument Name:	377 # 97042603
Collect Vers.:	2.1

Data Analysis

Base Call Start:	1044
Base Call End:	10616
Primer Peak Loc.:	1044
Signal:	G (373), A (239), T (218), C (307)
Matrix Name:	dRhodamine Matrix
Channels Ave.:	3
Basecaller:	ABI100
Basecaller Version:	Version 3.0
Base Spacing Used:	9.90
Base Spacing Calculated:	9.90

```
   1  GCGANTTGNG TACCGGGCCC CCCCTCGAGG TTTTTTTTTT TTTTTTGTT TCCAGATGTT   60
  61  TTATTGATGT TAAACAGACA TGGATATTCG GTGTCTGGTT TAATCAAACA GGCCGAAGCC  120
 121  CATGTCTTCA TCAGACTCCT CAGATCCTCC TTGGGTGCCT CCTCTTTAGC CGCAGGAGCA  180
 181  GCGGATTTCT GCTCTGTAGC TGCCGCAACA GGGGCTGCAA CAGCGAAAGC GGTGGGATCA  240
 241  GCCAGGTAGG CCTTCACCTT CTCAGCCAAG GGGAATGTGT AGTCTGTTTC GACAGTGACA  300
 301  GCCAGGACCC TCTTGTATCC ATTGATGATA GTGTGAGGGA TGGAAGCAAG AGTTGGGTAG  360
 361  CCGATCTGCA GACACACACT GGCGATGTTC CTACACCCTT CAGGAACCTN TTGTGCAGGG  420
 421  CGTCCTCAGT GATGTCCAGC ACCTCGGGGC TGTAGACACT GCCGTTATCA TACACCTGCT  480
 481  GGATGATCAA GCCCGTAGGA GAAGGGCGAG ATGTTCAGCA TGTTCAGCAA CCGTGGCCTC  540
 541  GCTTGGCGCC CACCTTGCTC CAGGTTTGAT AACTGAACGT ACTCAAGATT TCAATGGTTC  600
 601  CTCTGGANAT CTTGGNGGNG ATTCCCAAAG CCTGGAANAA AAAGGTNTTT TNAGGACCGA  660
 661  CCCCGGGGTT TTGGGCCGGC CAANTNACTT NACAGGGGGC AATGGCCCAN CCCGGGCANN  720
 721  ANNGGGCNCT TTGTTTGCCA NCANNAGNNT TNGGNCCTAA NNNAAANCCT CTTGGGGAAA  780
 781  ACAAANCCCC NTTTCCCNGG NTTNTGGGAA ANNACCCTTT CCAAANTGGG TNNNTTTCCA  840
 841  GGGGGCNCNA ANGGCCTTCN NAAAAANGGG GGTTTTCCCC TNNAACCCAA NGGNTTTCCC  900
 901  CCNNGGGACN ANCNGNNGGG NNNNANTTNN TTNGNNCCCA NTTTTTNGNC CCCNNNNAAA  960
 961  CCTTNGGGAA NNNTCCCCCN NNGGGNNNTT TNNAAAAAAN NGGGNNTTNC NNGGGNCCNN 1020
1021  TTTNCNGGGG G                                                      1080
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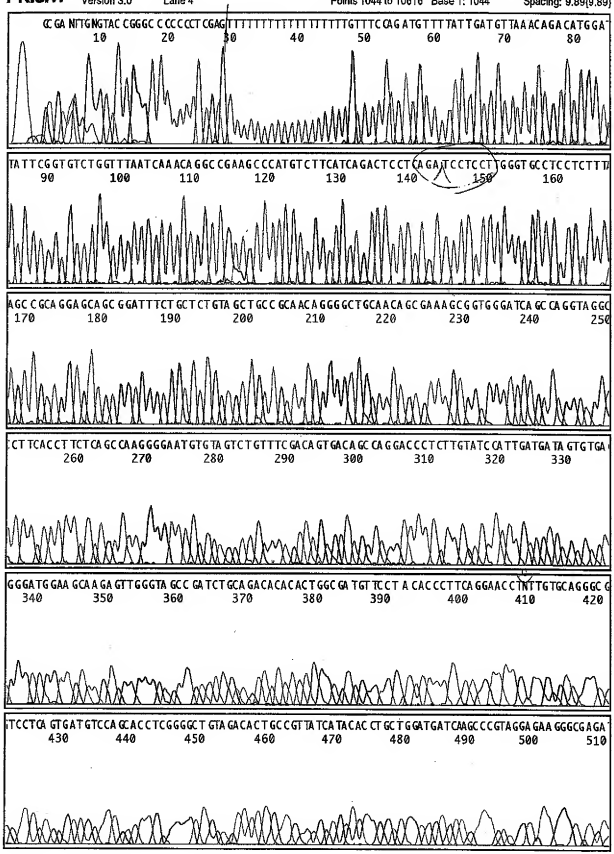
Version 3.0
ABI100
Version 3.0
A150T7
Lane 4
DT (BD Set Any-Primer)
dRhodamine Matrix
Points 1044 to 10616 Base 1: 1044
Mon, Jul 20, 1998 11:40 PM
Mon, Jul 20, 1998 2:33 PM
Spacing: 9.89[9.89]
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90 100 110 120 130 140 150 160
AGCCGCAGGAGCAGCGGATTTCTGCTCTGTAGCTGCCGCAACAGGGCTGCAACACGCGAAAGCGGTGGGATCAGCCAGGTAGGC
170 180 190 200 210 220 230 240 250
CTTCACCTTCTCAGCCAAGGGGAATGTGTAGTCTGTTTCGACAGTGACAGCCAGGACCCTCTTGTATCCATTGATGATAGTGTGA
260 270 280 290 300 310 320 330
GGGATGGAAGCAAGAGTTGGGTAGCCGATCTGCAGACACACACTGGCGATGTTCCTACACCCTTCAGGAACCTNTTGTGCAGGGCG
340 350 360 370 380 390 400 410 420
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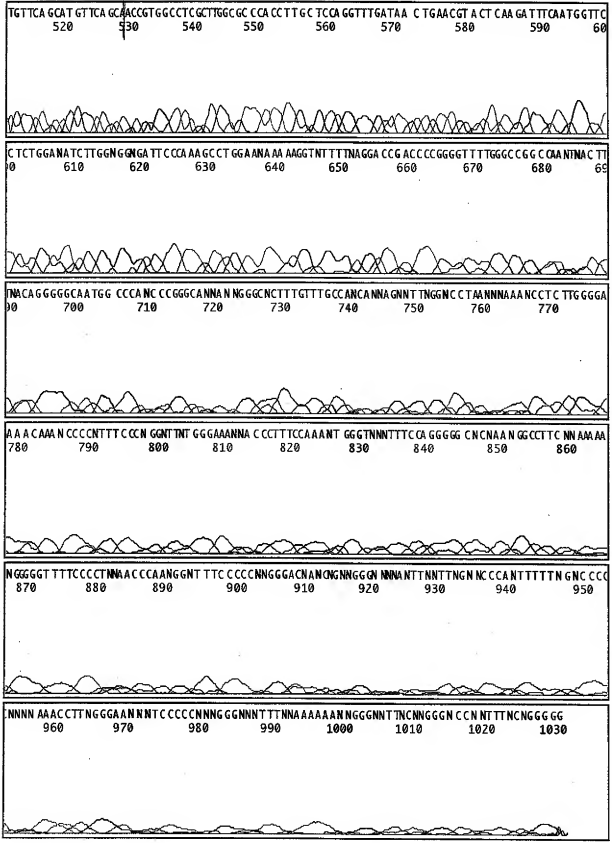
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520 530 540 550 560 570 580 590 60
C TCT GGANA TC TTGGNGGNGA TT CCCA AA GCCT GGA ANA AA AAGGTNT TTTNAGGA CCG ACC CC GGGGT TTTGGGCC GG C CAA NTNA C TT
0 610 620 630 640 650 660 670 680 69
TNACA GGGGGCAA TGG CCCA NC CC GGGCANNA N NGGGCNCTTTGTTTGCCANCANNAGNNTTNGGNC C TAANNNAAANCCTC TTGGGGA
00 700 710 720 730 740 750 760 770
A A A CAAA N CCCCNTTTCCCN GGNTTNT GGGAAANNA CCCTTTCCAAANT GGGTNNNTTTCCA GGGGG CNCNAA N GGCCTTC NN AAAAA
780 790 800 810 820 830 840 850 860
N GGGGGT TTTCCCCTNNA A CCAANGGNT TTC CCCCNNGGGACNANCNGNNGGGN NNNANTTNNTTNGN NC CCA NTTTTTTN GNC CCC
870 880 890 900 910 920 930 940 950
NNNNN AAACCTTNGGAANNNTCCCCCNNNGGGNNNTTTNNAAAAAANNGGGNNTTNCNNGGGN C CN NTTTNCNGGGGG
960 970 980 990 1000 1010 1020 1030

Data Collection

File:	06*PARPm2
Sample:	PARPm2
Comment:	
Lane Number:	6
Channel Number:	37
Number of Scans:	10620
No. of Channels:	194
Length:	1057
Run started at:	8/9/1998, 15:34
Run stopped at:	9/9/1998, 00:35
Gel:	Gel File
Dyeset/Primer:	DT {BD Set Any-Primer}
Comb:	
Instrument Name:	377 # 97042603
Collect Vers.:	2.1

Data Analysis

Base Call Start:	894
Base Call End:	10620
Primer Peak Loc.:	894
Signal:	G (295), A (364), T (336), C (307)
Matrix Name:	dRhodamine Matrix
Channels Ave.:	3
Basecaller:	ABI100
Basecaller Version:	Version 3.0
Base Spacing Used:	9.67
Base Spacing Calculated:	9.67

```
   1  CTTTGAATNC CCTTCTCGTG ACCTCTTATG ATGTTATTTA TTAACACATT TTAAGAAACC   60
  61  GCCACAAGAG CGGCGTCAGC CTTACCTCAC GGTAGGGACG CGTGAAAGAA AGAGGCCGCG  120
 121  AAAGGAAGCG GCACAATATT AGTATCAGTC GATCATCCAA TCAGAATCGC GCGGAAAATG  180
 181  CGTATCTGTG CGCTGATTGG TCAATGCACG TGTCTGTCAC GGTCAGTGCG TATTTGTACA  240
 241  GTCGCACTTA GAAAGGATAC AGCTGCGGTC AATATAGCAT CATTCTGTGA CGCTATAAAA  300
 301  CAATAAATAA TATTTTTACA TTACTGTGAG GGTTGAGTTT ACGGTTGGGG TAATAAAATA  360
 361  CAAATAATGG ATAATTTAAT AGCAAAGAAT GTAAATAGTT CCCGTTAACT TTCGGCCGCA  420
 421  GATGTATCCC TTCTAACAAC AGCCAAAAAT CTGCACAGAA CCAAAATCAC TGTGCATCTA  480
 481  AATTCATCGC AATACCTTTA TTTTAATGAA TGTATTTTAA TGAATTGACC ATTAATAGGG  540
 541  AAATTTGGTC CGCCAAAAAA GTCACTTTAT TATCTATTTT GATGTTTTTC ATCTTATTTT  600
 601  TTTGTCTAAA CTGACTTTTA TCATCTTGAC AATCATTTTT CTATTTTCTG CCATTTTTTA  660
 661  AATTGAACAT CTTATAATAA AACTCTTTCT CTTTCTATAT AAAATTCGTT TATTGCAATA  720
 721  ACTAAGAATA CTGTAAACAA ATTTGNATTA TTTCTATATA TTTTTATAAA AATAATACTT  780
 781  ACCAAATGTA AAATTNTCCA CACAAATTAA AATGATCGGG TTTGGTTTTA ATTCGTAACG  840
 841  CATATATATT TTAAGGGTTC TTAATACNGA TGAATTCAAC TTGTTCCCAG CATTGGTCAN  900
 901  ANCCNTATGC TAGTNGAANC CTTTAAAGTC GACCTGGAGG CTTGCAACTT TTCCCTTTAA  960
 961  GGGGGGNGNN TTAAAACTTG GGGNAAANNA NGGNAAAANN GGNTCCCCGG GGNGAAAANT 1020
1021  NTNNCCNNNC CAAATTCCCC CAAANTTNNG NCGGGGG                          1080
```

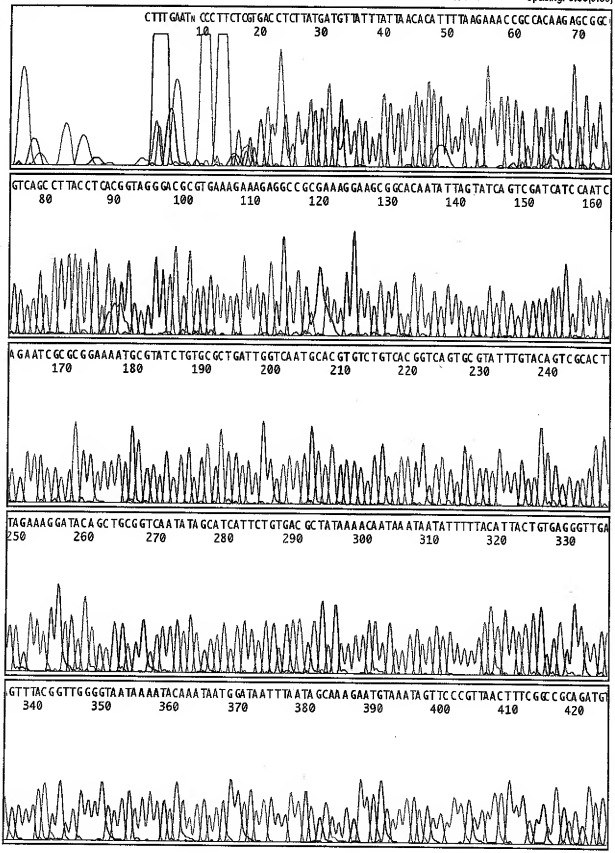
ABI PRISM™

Version 3.0
ABI100
Version 3.0

PARPm2
Lane 6

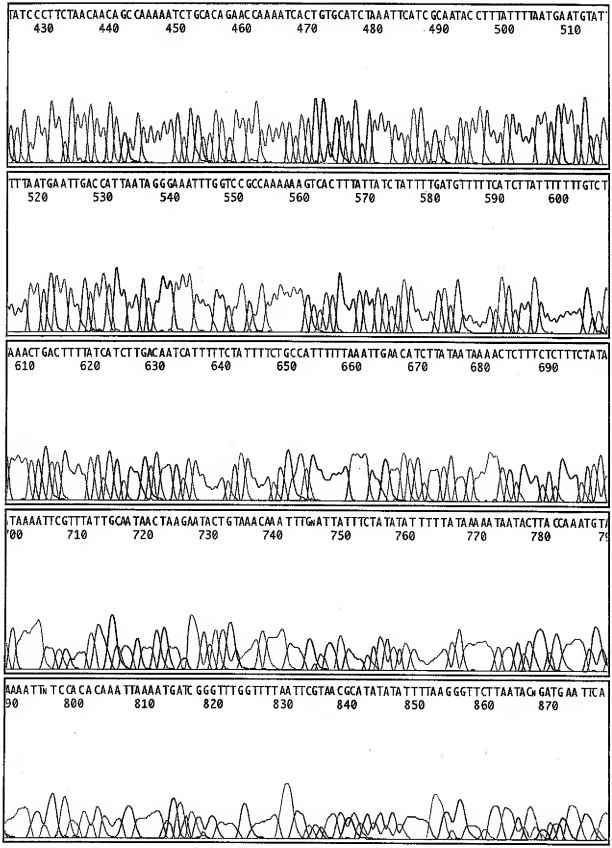
DT (BD Set Any-Primer)
dRhodamine Matrix
Points 894 to 10620 Base 1: 894

Wed, Sep 9, 1998 9:13 AM
Tue, Sep 8, 1998 3:34 PM
Spacing: 9.66[9.66]



ABI PRISM™

Version 3.0		DT (BD Set Any-Primer)	Wed, Sep 9, 1998 9:13 AM
ABI100	PARPm2	dRhodamine Matrix	Tue, Sep 8, 1998 3:34 PM
Version 3.0	Lane 6	Points 894 to 10620 Base 1: 894	Spacing: 9.66{9.66}



Data Collection

File:	14*ARPm
Sample:	ARPm
Comment:	
Lane Number:	14
Channel Number:	71
Number of Scans:	10624
No. of Channels:	194
Length:	1083
Run started at:	29/8/1998, 15:54
Run stopped at:	30/8/1998, 00:55
Gel:	Gel File
Dyeset/Primer:	DT {BD Set Any-Primer}
Comb:	
Instrument Name:	377 # 97042603
Collect Vers.:	2.1

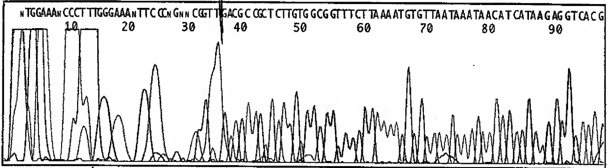
Data Analysis

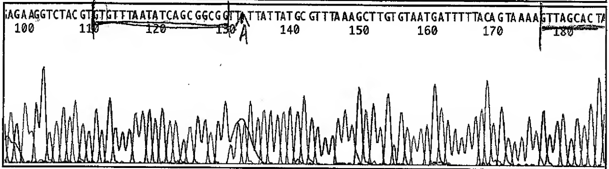
Base Call Start:	938
Base Call End:	10624
Primer Peak Loc.:	938
Signal:	G (361), A (349), T (375), C (273)
Matrix Name:	dRhodamine Matrix
Channels Ave.:	3
Basecaller:	ABI100
Basecaller Version:	Version 3.0
Base Spacing Used:	9.49
Base Spacing Calculated:	9.49

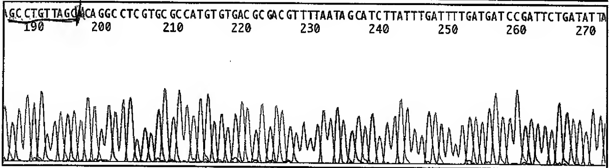
```
   1  NTGGAAANCC CTTTGGGAAA NTTCCCNGNN CGGTTTGACG CCGCTCTTGT GGCGGTTTCT  60
  61  TAAAATGTGT TAATAAATAA CATCATAAGA GGTCACGAGA AGGTCTACGT GTGTTTAATA 120
 121  TCAGCGGCGG TTNTTATTAT GCGTTTAAAG CTTGTGTAAT GATTTTTACA GTAAAAGTTA 180
 181  GCACTAGCCT GTTAGCACAG GCCTCGTGCG CCATGTGTGA CGCGACGTTT TAATAGCATC 240
 241  TTATTTGATT TTGATGATCC GATTCTGATA TTAATCATAT TTATGCGTAA AATGTGTGAT 300
 301  GGGTCTGCTA GTGGACATTA CATGCTAGTA CTTGTGCTAG TCGGTCGATC CACATTGAGA 360
 361  TGTTGCGCTA TTTGCCATTT TAAAACCAGT TACTCTCATT TTAGTGAAAT ATTCTTAAGC 420
 421  CACTAAGTTA AAATTTGTCA ATCACATATA ATTGTGTTTA TGTTTTATTT GAGTCATCAT 480
 481  ACCAGGTAAT AGTTTTATTT ATATTAGTAT GTACAATTTG GCATAAACTG CCTTCGGTTT 540
 541  TGATTGACAT CTACTTTGTA AAGTTAATCT TAAAGGGGTA AAGGCTCACC CAAAAGACAA 600
 601  TTCACCGTCA AGTGTTTTCA AATCTTATGA GTTTCTTAAT GAACATGGTA TGTTTTGGAG 660
 661  AAAACTGGAA ACCAACTACC ATAATACAAA TACAGGAAAA ATATACTATA GAAGTCGATG 720
 721  GTTACAGGTT TTCTGCATTC AAAATATCTA CACAAGTGTT TAATGGAAGG ACTCAAGTGA 780
 781  TTTGAAAAGG TAAGGGTGCA TAAATCAGTT TCATTTGGGT GAACTGNCTC TAAACATTTG 840
 841  GANTTTAGAC ACCTTAAGGC CATGGGCANC CAAGCTTGGT CCTTGGANGG GCCANTGNCC 900
 901  TACAGATTTA ACTTCCANCC TAATTAACCC CNCTTGACCA AGCTAATCAN GGCTTACTAN 960
 961  GNTTGGTTGG AACATCCNGC NGGTGTGTTN ATNCCAGAAA NACTTAACCC TGCGGGCCAT 1020
1021  GGGCCACCNG GATTGGGGAC CCTGCCTTAA NCCTTNCAAA GGCTTTTGGG TTNAAAAAGG 1080
1081  GCC                                                              1140
```

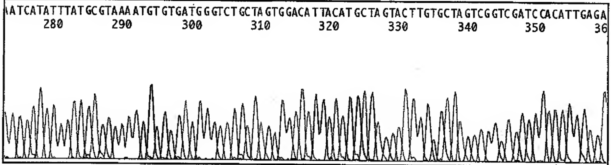
ABI PRISM

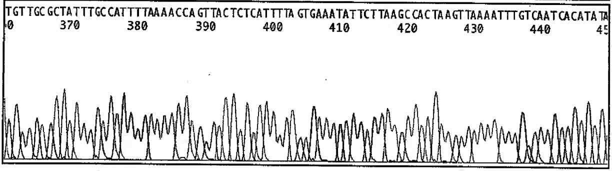
Model 377	14-ARPm	Signal G:381 A:349 T:375 C:273	Page 2 of 4
Version 3.0		DT {BD Set Any-Primer}	Sun, Aug 30, 1998 12:40 PM
ABI100	ARPm	dRhodamine Matrix	Sat, Aug 29, 1998 3:54 PM
Version 3.0	Lane 14	Points 938 to 10624 Base 1: 938	Spacing: 9.49(9.49)

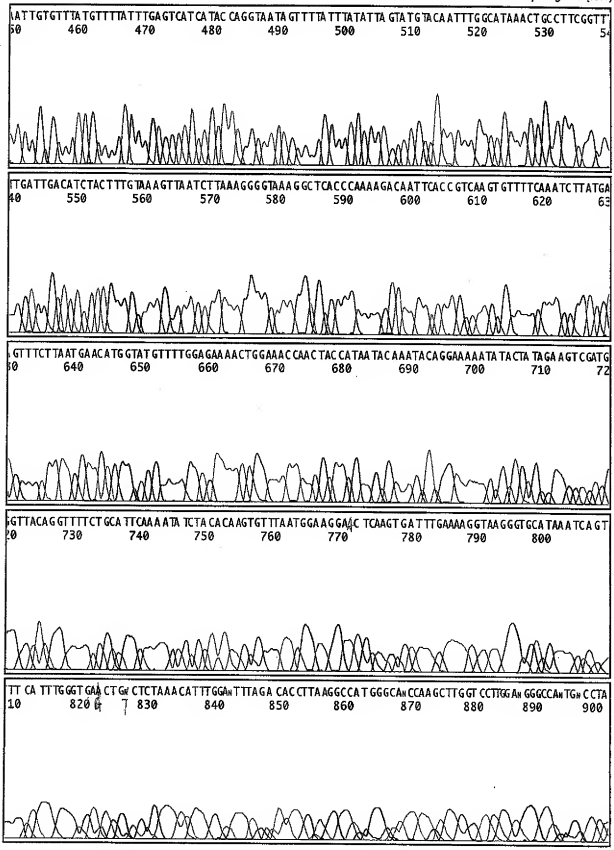








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470
480
490
500
510
520
530
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550
560
570
580
590
600
610
620
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640
650
660
670
680
690
700
710
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730
740
750
760
770
780
790
800
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820
830
840
850
860
870
880
890
900

Data Collection

File:	10*ARPP-M13
Sample:	ARPP-M13
Comment:	
Lane Number:	10
Channel Number:	53
Number of Scans:	10620
No. of Channels:	194
Length:	1043
Run started at:	22/8/1998, 20:25
Run stopped at:	23/8/1998, 05:42
Gel:	Gel File
Dyeset/Primer:	DT {BD Set Any-Primer}
Comb:	
Instrument Name:	377 # 97042603
Collect Vers.:	2.1

Data Analysis

Base Call Start:	1167
Base Call End:	10620
Primer Peak Loc.:	1167
Signal:	G (253), A (227), T (209), C (218)
Matrix Name:	dRhodamine Matrix
Channels Ave.:	3
Basecaller:	ABI100
Basecaller Version:	Version 3.0
Base Spacing Used:	9.73
Base Spacing Calculated:	9.73

```
   1  GATTCCGCTC GGTACCCGGG GATCCGATTG GATCCCTTCC AAGGATCGGT GAACAGCCGG   60
  61  TTTAAAGACA AGGTTGTAAA ATCTGAGGAT TACAAACACA AGCAATACTT TTACACCTGC  120
 121  CTTGTGTGCA TCTTATTAAA TGACAAAATA TGAATTACTG TTTGTAAACT GTTTCCAGGT  180
 181  AATTTGGTTG CCTATTATAA GCTTTCAAAT ATTTAAAGAT AATCAGAGCA CTATTGTATT  240
 241  TGATTTACTG CTATAAACGT GTCTGAGACC AGCGTCCCTG CAAGTCATGA GATGCAGACA  300
 301  GACAGGGATA GAAGGAATCA TGTTCATTTG TGCAGTGGGA ACGACGAACA AGCACTGCAA  360
 361  CAGCCTGTCC ATAACTGAAC CTGCACATTT CTTAATACCA TAATGCATTT GTGATGGCTT  420
 421  GAGGCAGGGG TCACCAATCC TGTTGGGCCA TTGTCCCTGC AGGGTTTAGC TCTATCTTGC  480
 481  ATCAACACAC CTGCCTGGAT GTTTCAAACA TACCTAGTAA GACCTTGATT AGCTTGTTCA  540
 541  GGTGTGTTTA ATTAGGGTTG GAGCTAAAAT CTGTAGGACA CTGGCCCTTC AGGAACAAGC  600
 601  TTGGTGACCA CTGCCTGAGG TGTCTAAATC AAATGTTTAG AGACAGCTCA CCCAAATGAA  660
 661  AACTGATTTA TGCCCCTTAA CTTTTCAAAT CACTTGAGTT CCTTNCATTA AACACTTGNG  720
 721  TAGATATTTT GAATGCAGAA AACCTGTACC ATCGACTTCT ATAGTATAAT TTTTCCTGGA  780
 781  TTTGGATTAT GGTAGTTGGG TTCCAAGTTT NTCCAAAACA TACCATGTTC ANTTAAGAAA  840
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 901  AAGAATAACT TTCCAAGGAG ATGTCAATTC AAAACCNAAN GCCNTTTTTC CCAAATGGGN  960
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1021  TTNTTTGGGA TTGGCAAATT TTN                                        1080
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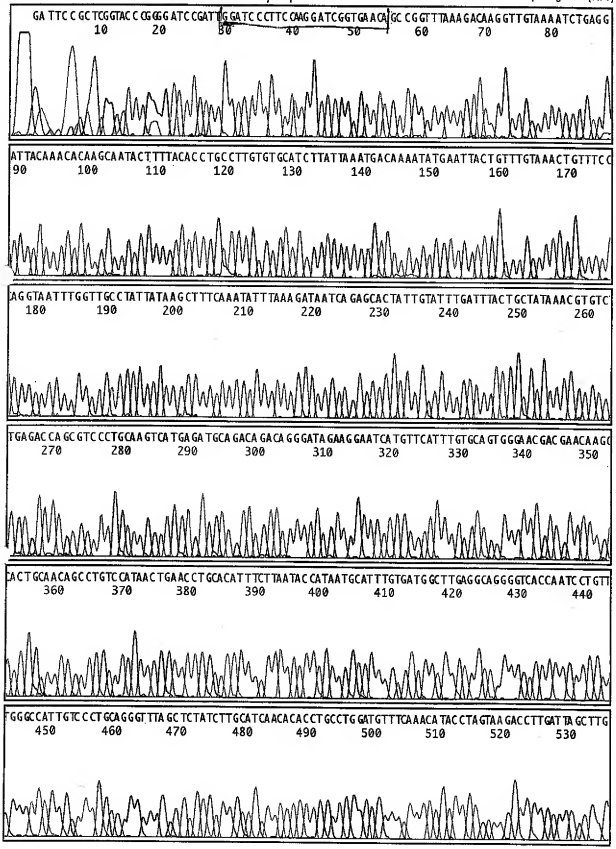
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180 190 200 210 220 230 240 250 260
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270 280 290 300 310 320 330 340 350
CACTGCAACAGCCTGTCCATAACTGAACCTGCACATTTCTTAATACCATAATGCATTTGTGATGGCTTGAGGCAGGGGTCACCAATCCTGTT
360 370 380 390 400 410 420 430 440
TGGGCCATTGTCCCTGCAGGGTTTAGCTCTATCTTGCATCAACACACCTGCCTGGATGTTTCAAACATACCTAGTAAGACCTTGATTAGCTTG
450 460 470 480 490 500 510 520 530

ABI PRISM

Model 377 Version 3.0
ABI100 Version 3.0
ARPP-M13
Lane 10

Signal G:203 A:227 T:209 C:216
DT (dR Set Any-Primer)
dRhodamine Matrix
Points 1167 to 10620 Base 1: 1167

Sun, Aug 23, 1998 1:57 PM
Sat, Aug 22, 1998 8:25 PM
Spacing: 9.73(9.73)

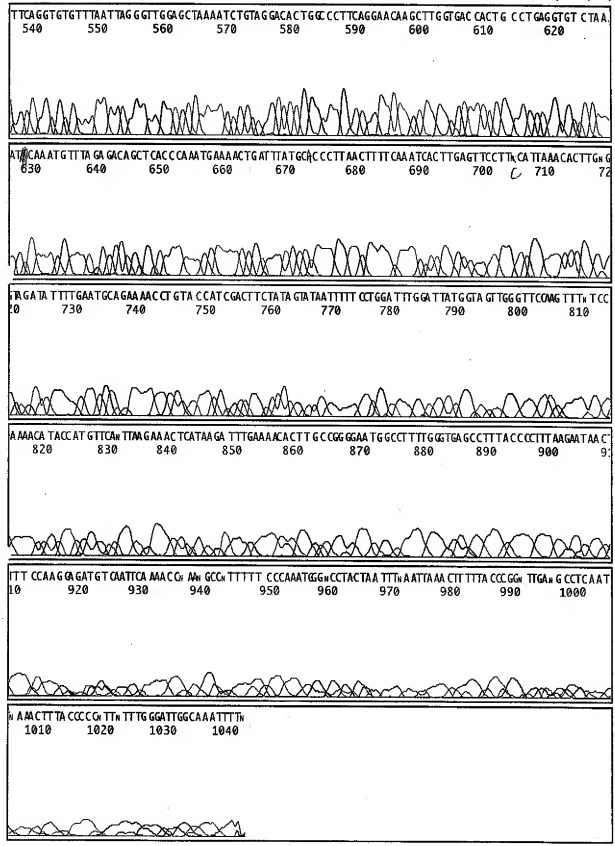


EXHIBIT 6

CLON MCKP fragments into CAT vector

5/2/98.

① Digestion with BamH I

	CAT3 vector (1ng/ml)	MCKP #3 - PT7 (reverse direction)	MCKP #5 - PT7 (reverse direction)
DNA	2	15	15
10X Buffer (for BamH1)	2	2	2
10X BSA	2	2	2
BamH1	1	1	1
H_2O	13	/	/

37°C incubation 2.5 hr. Run 0.5 ml each on gel

for CAT3 digestion, add CIAP 1.6 ml
10xCIAP buffer 2.4 ml
37°C incubation for another 1 hr.

② Run agarose gel.
Gel purification in 20 ml H_2O.

③ Ligation

CAT3 (dephosphorylated)	6 ml
DNA (#3, #5)	10 ml
5X ligase buffer	3 ml
T4 ligase	1 ml / 1.5 ml

16°C incubation overnight.

6/21 98

Transform 7.5 ml ligation reaction.

8/2/98.

① PCR colony screening of the MCKp23-CAT, MCKp5-CAT

for 1

10x PCR buffer	3
dNTP	1.5
$MgCl_2$	1.8
M13	0.3
MCK#	0.3
Taq	0.1
H_2O	23

MCKp23-CAT

using ligation reaction as positive control.

MCKp5-CAT

② Inoculation MCKp23-CAT #17 #19

MCKp5-CAT #19. #22.

in 6 ml LB/Amp. 37°C incubation overnight.

9/2/98.

① Digestion with BamHI and EcoRI

	pEGFP vector (0.745ug/ml)	MCKp #23-pT7 (reverse direction)	MCKp #5-pT7 (reverse direction)
DNA	3	11.5	14.5
10xBuffer 2	2	2	2
10xBSA	2	2	2
BamHI (20u/ml)	1	1	1
EcoRI (80u/ml)	0.5	0.5	0.5
H_2O	11.5	3	/

pEGFP MCK23 MCK5

37°C incubation ~~overnight~~ for 2 hrs.

run 0.5 ml on agarose gel.

② Gel purify the digested fragments.
The vector is smeared.

for MCK23 B1/E1 fragment and
MCK5 B1/E1 fragment,
purify in 30 ml HW

③ Ligation with pGFP E1/B1 (dephosphorated fragment)

DNA	10.5
5X ligase buffer	3
T4 ligase	1
GFP vector	0.5
~~15~~ μl	16°C incubation overnight

10/2/98.
Transform 10 ml ligation reaction with 100 ml competent cell.

11/2/98
Inoculation MCKp23-GFP #1. #2. #3. #4 pEGFP vector.
MCKP5-GFP #1 #2. #3. #4.
in 4 ml LB / Kanamycin (30 μg/ml)

12/2/98.
① plasmid miniprep. in 30 ml HW

② Single digestion with BamH1.

DNA (plasmid)	1 ml	37°C incubation
10X Buffer	1 ml	1 hr.
10 X BSA	1 ml	

MUKP23-GFP 1 2 3 4 — MUKP5-GFP 1 2 3 4

13/2/98.

① Double digestion with BamH1 and EcoR1.
37°c incubation 1 hr. (using BamH1 buffer)

② DNA concentration (50x dilution)

	23①	23②	23③	23④	5①	5②	5③	5④
A_{280}	0.115	0.114	0.125	0.085	0.236	0.170	0.207	0.221
A_1/A_2	1.9312	1.9339	1.9961	2.0174	1.8980	1.903	1.9070	1.9094
[DNA] (ug/ml)	0.2875	0.285	0.3125	0.2125	0.59	0.425	0.5175	0.5525.

③ choose 23① and 5① to do microinjection.
dilute the two plasmids to 0.2ug/ml.

20/2/98

~~Ligation~~ clon MUKP23 and MUKP5 into pEGFP vector

① Digest with BamH1 / EcoR1

DNA (MUKP23-pT7.) MUKP5-pT7	14.5ul
10xBuffer	2 ul
10XBSA	2 ul
BamH1	1ul
EcoR1	0.5 ul
	20ul.

37°c incubation for 2hr

(before ligation 1ul/Lane)

② Gel purification into 20 ul H_2O

③ ~~Digest~~ Ligation

DNA (MUKP23. MUKP5) 6.5ul
pEGFP (BamH1/EcoR1 digested) 0.5ul

21/2/98.

Transformation 5 ml of each ligation reaction into 10 ml DH5α.
plating on LB / kanamycin plate.

22/2/98

Inoculation. { MCKp23 - EGFp #1. #2.
MCKp5 - EGFp #1. #2
in 3 ml LB / kanamycin.

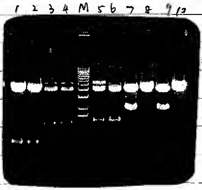
Inoculation ARPp0.8kb - EGFp #1 #2.
MLCp2kb - EGFp #1 #2. #3
MLCp3kb - EGFp #1.

23/2/98.

① Plasmid miniprep in 30 ml H₂O

② Digestion.

lane 1 ARPP-EGFp (0.8kb) #1. EcoRI/BamHI
2. " " #2 " "
3. MCKp23-EGFp (1.4kb) #2. " "
4 " " #1 "
5. MCKp5-EGFp (1.5kb) #1. "
6. MCKp5-EGFp (1.5kb) #2 "
7. MLCp2kb-EGFp #1. BamHI / HindIII (~~BamHI / HindIII~~)
8. " #2 "
9 " #3 "
10 MLCp3kb-EGFp #1 "



③ Check concentration (100X dilution)

ARPP 0.8kb-EGFp #1. MCKp23-EGFp #2. MCKp5-EGFp #2. MLCp2kb-EGFp #1

3/3/98.

check the concentration of MCK5-EGFP (for deletion)

#1. ~0.5 ug/ml

#2 ~0.8 ug/ml

13/3/98.

Nested deletion of MCK5-EGFP

① Single digestion by XhoI

DNA (MCK5-EGFP #2)	3.5 ml
10 × Promega buffer D	1 ml
XhoI (Promega, 40u/ml)	0.5 ml
100 × BSA	0.1 ml
	10 ml

37°C incubation 1 hr

check 1ml on agarose gel

② Modified by Klenow

XhoI digested DNA	7 ml
10 × Klenow buffer	1 ml
dNTP	1 ml
Klenow (1 u/ml)	1 ml
	10 ml

37°C 15' → 65°C 30'

③ Add 2vol NaAc/Glycogen, 7.5ml ethanol. -80°C for 30'

14000rpm × 20' at 4°C wash with 1.80 ml 70% ethanol 14000rpm x10' at 4°C

air dry. redissolve in 15 ml H_2O

④ Secondary digestion by HindIII

modified DNA	15 ml
HindIII (10 u/ml)	2 ml
10 × Promega buffer	2 ml

④ mix the following: S1 buffer 16.5 ml
dH₂O 33 ml
S1 nuclease 0.5 ml
50 ul

aliquot 3 ml into 16 tubes. place on ice

⑤ Exo III buffer 8 ml
0.3 M NaOH 4 ml
H₂O 12 ml
24 ml

mix 2 ml with 2 ml double digested DNA
equilibrate at 25°C for 3'

⑥ Remove 2 ml as time = 0 sample, mix with 3 ml S1 nuclease buffer
put on ice.

⑦ Add 1 ml Exo III to the reaction tube, incubation at 25°C
time interval = 5 min. remove 2 ml at each time point. mix with
3 ml S1 nuclease buffer. put on ice

⑧ Incubation at RT for 30'

⑨ Add 1 ml S1 stop solution. 65°C 10'
[] made a mistake at this step. put on 65°C first, then add stop solution

⑩ check 7 ml on agarose gel

6). mix the following

10 x ligation buffer	4 ul
25% PEG	8 ul
T4 DNA ligase	2 ul
ddH2O	18 ul
	32 ul

Add 17 ul to each of 3 ul ~~solution~~ tube. 16°C incubation overnight.

14/03/98.

Transformation

transform 10 ul of ligation (t=1, 3, 5, 7, 9, 11, 13, 15) with 100 ul competant cell

18/03/98

PCR check deletion. (only 13 colonies grow from the 16 plates?

use EGFP-1 primer and nick4 primer.

Positive control: MCK5-EGFP Plasmid.

with insert: #3 #5 #6 #7

#9. #10 #12

17/03.

Inoculation: MCK5-EGFP deletion #3. #5. #6. #7. #9. #10. #12

in 5 ml LB/ Kanamycin media

18/03.

23/03

Measure DNA concentration for MCK5-EGFP nested deletion constructs. (50 X dilution)

	#3	#5	#6	#7	#9	#10	#12
A260	0.275	0.282	0.267	0.255	0.194	0.492	0.295
A280	0.123	0.152	0.185	0.195	0.145	0.268	0.161
A1/A2	1.8312	1.8511	1.8368	1.8198	1.8660	1.8368	1.8338
[DNA] (ng/μL)	0.6875	0.705	0.6675	0.8875	0.485	1.23	0.7375

24/03

Because the MCK5-CAT construct I've already got was not correct

Try PCR to screen the MCK5-CAT ligation plate again.

(Spot 89 colonies from 9/10 plate onto another new plate)

No.1 ~ No. 89

PCR use M13, MCK4 primers

Template No 20 — No 45 colonies

Positive colonies: No 28.

No 40

26/03.

① PCR colony screening again.

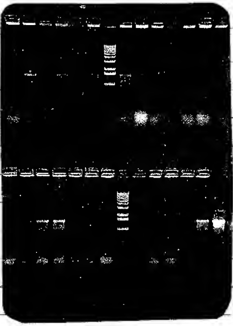
M13. MCK4 primers.

Template No 41 — No 70 colonies

positive colonies: #47 #49 #51 #52 #61 #62 #70

② Inoculating #28 #40 #47 #49

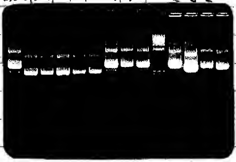
#51 #52 #61 #62 #70 in 5ml LB/Amp



27/04.

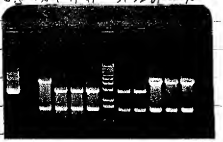
① Plasmid Miniprep MCKp5-EGFP CMV-EGFP. MLCp12-EGFP. ARPP0.8-EGFP
using QIAGEN Kit.
dissolve DNA in 10 ml PBS.
Run 0.5 ml on Agarose gel.

② Plasmid miniprep MCK5-CAT #38. #40. #47 #49, #51 #52. #61 #62. #70
in 30 ml H₂O.
Run 1 ml each on agarose gel.



③ Check MCK5-CAT plasmids
by restriction digestion (BamH1)

DNA	1 ml
10x buffer	1 ml
10x BSA	1 ml
BamH1	0.5 ml
H₂O	6.5 ml



16/05

Transformation { MCK5-CAT #38
MCK27-CAT #17

17/05.

Inoculation 3 colonies ~~each~~ from both of the transformation plate in 4ml LB/Amp

18/05.

① Plasmid miniprep MCK5-CAT ① ② ③
MCK27-CAT ① ② ③.

② Digestion with BamHI.
Ark with insert (correct!

③ Inoculate MCK5-CAT ① and MCK27-CAT ① in 100 ml LB/Amp
37°C incubation overnight.

19/05

Plasmid Maxiprep MCK5-CAT and MCK27-CAT
in 30 ml PBS
Check concentration 100 x fold dilution.

MCK5-CAT $A_1 = 0.132$ [DNA] = 0.66 μg/ml,
$A_1/A_2 = 1.74$

22/05.

① Inoculate MCK5-CAT and MCK27-CAT in 2ml of LB/Amp
250 rpm, 37°C for 8hrs.

2/15

① Plasmid Maxiprep NLK5-CAT and NLK22-CAT
Dissolve the DNA in 100 μl PBS

② check concentration (150 x dilution)

	NLK5-CAT	NLK22-CAT
A_1	0.774	1.130
A_2	0.420	0.601
A_1/A_2	1.8011	1.8802
[DNA]	5.8 μg/ml	8.4 μg/ml

③ Dilute into 1 μg/ml solution (150 μl each)

NLK5-CAT	8.62 μl + 141.38 μl PBS
NLK22-CAT	5.95 μl + 144.05 μl PBS

		CPM 1	
	N_1	45.70	47
	N_2	48.30	
MZF2-79-CAT	1	244.4	
	2	394.7	
	3	322.9	
	4	551.3	
	5	439.3	279.4
	6	148.2	
	7	206.4	
	8.	304.1	
MLCP-1-1-CAT	1	484.0 (437)	
	2	523.1 (476.1)	
	3	396.2 (349.2)	
	4	491.4 (444.4)	431.4
	5	73.3	
	6	429.4 (382.4)	
	7	484.6 (437.6)	
	9	540.2 (493.2)	

		CPM 1	
MCK23-CAT	1	358.3 (311.3)	
	2	76.4	
	3	47.8	
	4	516.8 (469.8)	
	5	393.5 (346.5)	319.6
	6	235.5 (188.5)	
	7	351.5 (304.5)	(74
	8	383.7 (336.7)	
	9	327.0 (280)	
MCK5-CAT	1	190.8 (143.8)	
	2	~~[illegible]~~ 503.7 (456.7)	
	3	58.2	
	4	221.8 (174.8)	
	5	100.7	
	6	309.8 (262.8)	
	7	220.9 (173.9)	215
	8	198.1 (151.1)	(4
	9	196.5 (149.5)	
	10	254.8 (207.8)	

CK60 239

~~357~~ { 281.2, 367 } { 98.5, 103.5, 229.7, 116.5 }

```
No. Target file  Definition  (05 bp                          Match% Over. INIT   OPT
  4 mck5-d3                                                   97.2  214  522   770
                 430        440        450        460        470        480
mckp1.5kb-1      TCCCTGAGTGAGAACATTGCATGTGCGCGTGACAGAAAACCAGAGATGGAAATACCTTCT
                 :::::::::::::::::::::::::::::: X:::::::::::::::::::::::::
mck5-d3          ACTAGCGGTGAGAACATTGCATGTGCGCGTGACAG-AAACCAGAGATGGAAATACCTTCT
                       10         20         30         40         50
                 490        500        510        520        530        540
mckp1.5kb-1      TTTGAATTGCATAATTGCTTAAAAGAAGACACAACAGGGATAGTTCACCCAAAAAACAGA
                 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
mck5-d3          TTTGAATTGCATAATTGCTTAAAAGAAGACACAACAGGGATAGTTCACCCAAAAAACAGA
                 60         70         80         90         100        110
                 550        560        570        580        590        600
mckp1.5kb-1      CCATTCTTTTTTTCTGTTGAACAAAAATTAAGATATTTTG-AAGAATGC-TTACCGAATA
                 :::::::::::::::::::::: :::::::::::::::::: :::::::: ::::::::::
mck5-d3          CCATTCTTTTTTTCTGTTGAAC-AAAATTAAGATATTTTGAAAGAATGCTTTACCGAATA
                120        130        140        150        160        170
                       610        620        630        640
mckp1.5kb-1      ACTTCCATATTTGGAAACTAATTACAGTGAAAGTCAATGGG
                 ::::::::::::::: :::::::::: :::::::::X
mck5-d3          ACTTCCATATTTGGAAGCTAATTACAGTG-AAGTCAATGGG
                180        190        200        210
```

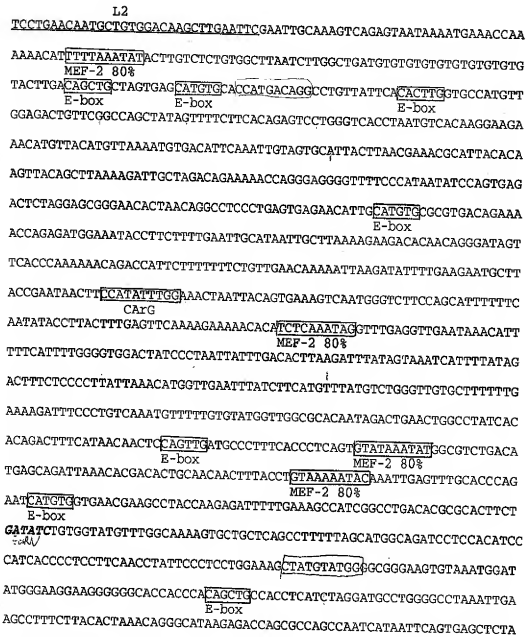
No. Target file Definition Match% Over. INIT OPT
2 mck5-d6 96.6 204 706 752

```
                670       680       690       700       710       720
mckp1.5kb-1 TATACCTTACTTTGAGTTCAAAAGAAAAACACATCTCAAATAGGTTTGAGGTTGAATAAA
            X:::::::::::::::::::::: : :::::::::::::::::::::::::::
mck5-d6     CGCTACCGGCTTTGAGTTCAAAAGAAAAACAAAACTCAAATAGGTTTGAGGTTGAATAAA
                10        20        30        40        50        60
                730       740       750       760       770       780
mckp1.5kb-1 CATTTTTCATTTTGGGGTGGACTATCCCTAATTATTTGACACTTAAGATTTATAGTAAAT
            :::::::::::::::::: ::::::::::::::::::::::::::::::::::::::
mck5-d6     CATTTTTCATTTTGGGGTGAACTATCCCTAATTATTTGACACTTAAGATTTATAGTAAAT
                70        80        90        100       110       120
                790       800       810       820       830       840
mckp1.5kb-1 CATTTTATAGACTTTCTCCCCTTATTAAACATGGTTGAATTTATCTTCATGTTTATGTCT
            :::::::::::::::::::::::::::::: :::::::::::::::::::::::::::
mck5-d6     CATTTTATAGACTTTCTCCCCTTATTAAACAAGGTTGAATTTATCTTCATGTTTATGTCT
                130       140       150       160       170       180
                850       860       870
mckp1.5kb-1 GGGTTGTGCTTTTTTGAAAAGATTTCCCTGTCA
            :::::::: ::^:v:::::::::: :::::::
mck5-d6     GGGTTGTG--TTTTTGAAAAGATTT-CCTGTCA
                190       200       210
```

No. Target file Definition Match% Over. INIT OPT
6 mck5-d7 94.6 168 384 600

```
          1320       1330       1340       1350       1360       1370
mckp1.5kb-1 AAATTGAAGCCTTTCTTACACTAAACAGGGCATAAGACCAGCGCCAGCCAATCATAAT
            : : X:::::::::::::::::::::::::::::::::::::::::::  ::::::::::
mck5-d7     CTACCGGACCCTTTCTTACACTAAACAGGGCATAAGACCAGCNCCAGCAATCATAAT
                  10         20         30         40         50         60
          1380       1390       1400       1410       1420       1430
mckp1.5kb-1 TCAGTGAGCTCTAAAATGGGCCAGCCAATGGCTGCAGGGGCTAGAGGTATATATATCCAA
            :::::::::: :::::::::::::::::::::::::::::::::::  ::: :::::::
mck5-d7     TCAGTGAGCTGTAAAATGGGCCAGCCAATGGCTGCAGGGGCTAGAGG--TAT-TATCCAA
                  70         80         90        100        110
          1440       1450       1460       1470       1480
mckp1.5kb-1 ATCAAACTCTTCTTGCTTGGGTGACCCCTATTTCGGCTTGGTGAACAGGATCCGG
            :::::::::::::::::::::::::::::::::::::::::::::::::::: ^
mck5-d7     ATCAAACTCTTCTTGCTTGGGTGACCCCTATTTCGGCTTGGTGAACAGGATCCAC
            120        130        140        150        160        170
```

MCKp-1


L2
MEF-2 80%
E-box
E-box
E-box
E-box
CArG
MEF-2 80%
E-box
MEF-2 80%
MEF-2 80%
E-box
TATA
MCK4 primer
MCK 3 primer
intron 1.6 kb
MCKP 2 primer
MCK1 primer

```
GAATTGCAAA GTCAGAGTAA TAAAATGAAA CCAAAAAACA TTTTTAAATA TACTTGTCTC
        70          80          90         100         110         120
TGTGGCTTAA TCTTGGCTGA TGTGTGTGTG TGTGTGTGTG TACTTGACAG CTGCTAGTGA
       130         140         150         160         170         180
GCATGTGCAC CATGACAGGC CTGTTATTCA CACTTGGTGC CATGTTGGAG ACTGTTCGGC
       190         200         210         220         230         240
CAGCTATAGT TTTCTTCACA GAGTCCTGGG TCACCTAATG TCACAAGGAA GAAACATGTT
       250         260         270         280         290         300
ACATGTTAAA ATGTGACATT CAAATTGTAG TGCATTACTT AACGAAACGC ATTACACAAG
       310         320         330         340         350         360
TTACAGCTTA AAAGATTGCT AGACAGAAAA ACCAGGGAGG GGTTTTCCCA TAATATCCAG
       370         380         390         400 d3      410         420
TGAGACTCTA GGAGCGGGAA CACTAACAGG CCTCCCTGAG TGAGAACATT GCATGTGCGC
       430         440         450         460         470         480
GTGACAGAAA ACCAGAGATG GAAATACCTT CTTTTGAATT GCATAATTGC TTAAAAGAAG
       490         500         510         520         530         540
ACACAACAGG GATAGTTCAC CCAAAAAACA GACCATTCTT TTTTCTGTT GAACAAAAAT
       550         560         570         580         590         600
TAAGATATTT TGAAGAATGC TTACCGAATA ACTTCCATAT TTGGAAACTA ATTACAGTGA
      ) 610         620         630         640 d6      650 d10     660
AAGTCAATGG GTCTTCCAGC ATTTTTTCAA TATACCTTAC TTTGAGTTCA AAAGAAAAAC
       670         680         690         700         710         720
ACATCTCAAA TAGGTTTGAG GTTGAATAAA CATTTTTCAT TTTGGGGTGG ACTATCCCTA
       730         740         750         760         770         780
ATTATTTGAC ACTTAAGATT TATAGTAAAT CATTTTATAG ACTTTCTCCC CTTATTAAAC
       790         800         810         820         830         840
ATGGTTGAAT TTATCTTCAT GTTTATGTCT GGGTTGTGCT TTTTTGAAAA GATTTCCCTG
       850         860 d8      870         880         890         900
TCAAATGTTT TTGTGTATGG TTGGCGCACA ATAGACTGAA CTGGCCTATC ACACAGACTT
       910         920         930         940         950         960
TCATAACAAC TCCAGTTGAT GCCCTTTCAC CCTCAGTGTA TAAATATGGC GTCTGACATG
       970         980         990        1000        1010        1020
AGCAGATTAA ACACGACACT GCAACAACTT TACCTGTAAA AATACAAATT GAGTTTGCAC
      1030        1040        1050        1060        1070 d12    1080
CCAGAATCAT GTGGTGAACG AAGCCTACCA AGAGATTTTT GAAAGCCATC GCCCTGACAC
      1090        1100        1110        1120        1130        1140
C  CACTTCT GATATCTGTG GTATGTTTGG CAAAAGTGCT GCTCAGCCTT TTTAGCATGG
      1150        1160        1170        1180        1190        1200
CAGATCCTCC ACATCCCATC ACCCCTCCTT CAACCTATTC CCTCCTGGAA AGCTATGTAT
      1210        1220        1230        1240        1250        1260
GGGGCGGGAA GTGTAAATGG ATATGGGAAG GAAGGGGGGC ACCACCCACA GCTGCCACCT
      1270        1280        1290 d7     1300        1310        1320
CATCTAGGAT GCCTGGGGCC TAAATTGAAG CCTTTCTTAC ACTAAACAGG GCATAAGAGA
      1330        1340        1350        1360        1370        1380
CCAGCGCCAG CCAATCATAA TTCAGTGAGC TCTAAAATGG GCCAGCCAAT GGCTGCAGGG
      1390        1400        1410        1420        1430        1440
GCTAGAGGTA TATATATCCA AATCAAACTC TTCTTGCTTG GGTGACCCCT ATTTCGGCTT
      1450        1460        1470        1480        1490        1500
GGTGAACAGG ATCCGG.... ..... ..... ..... ..... .....
```

d4 d9 d11

mck4 primer

EXHIBIT 7

7/10

Nested deletion of pMLC 2kb-EGFP

① pMLC2kb-EGFP @ #2 XhoI digestion

plasmid	8.5 ml
10X promega D	1 ml
100X BSA	0.1 ml
XhoI (40u/ml)	0.5 ml
	10 ml

37°C incubation for 2hr.

run 1 ml on agarose gel

② nuclify by klenow

XhoI digested DNA	7 ml
10 x klenow buffer	1 ml
dNTP mix	1 ml
diluted klenow (0.5u/ml)	1 ml
	10 ml

37°C 15' → 65°C 20'

③ Add NaCl / Glycogen 30 ml. 75 ml of ethanol

-20°C overnight.

8/10

④ precipitate the DNA by 14000 rpm x20', wash with 70% ethanol 14000 rpm x10', dry. redissolve in 10 ml H_2O

⑤ Hind III digestion

DNA	10 ml
10 x buffer NEB 2	2 ml (50mM NaCl)
HindIII (20u/ml)	1 ml
H_2O	8 ml
	21 ml

37°C incubation for 1hr

check 1 ml on agarose gel

④ Mix the following: S1 buffer 165 ml
dH2O 33 ml
S1 nuclease 0.5 ml / 5 ml

aliquot 3 ml into 16 tubes, put on ice.

⑤ Exo III buffer 8 ml
0.3 M NaCl 8 ml
H2O 8 ml / 24 ml

mix 20 ml with 20 ml double digested DNA
equilibrate at 25°C for 3'

⑥ Remove 2 ml as time = 0 sample. Mix with 3 ml S1 nuclease/buffer put on ice.

⑦ Add 1 ml of Exo III to the tube incubate at 25°C
time interval = 6 min, remove 2 ml at each time point,
mix with 3 ml S1 nuclease buffer. put on ice

⑧ incubate at RT for 30'

⑨ Add 1 ml S1 stop solution, 65°C 10'

⑩ check 3 ml on agarose gel.

M 0 1 15 CM

9/10 ② 10 x ligation buffer 20 ml
20% PEG 40 ml
T4 ligase 1 ml
HW 109 ml / 170 M

Mix 17 ml with Time = 1, to Tm = 10. tubes.
RT for 2hrs.

③ Transforme half the ligase reaction (10 ml) of T=1, 3, 5, 7, 9.

9/10.
④ PCR screening the colonies
by EGFP-1 primer and M2 primer

(⑤. Inoculate
5 (2.0Kb)
8 (1.8Kb)
13 (1.5Kb)
81 # 12 (1.3Kb)
15 (1.0Kb)
28 (0.8Kb)

10/10
① Plasmid miniprep.

② Measure concentration (150 X dilution)
A260 A260/A280 [DNA] (ug/ml) [DNA]
#5 0.0091 1.7576 0.75 #31 0.0100 1.5227 0.75

15/12

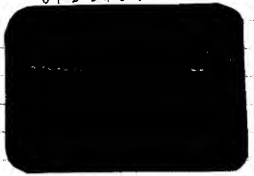
· ARPP2.1-EGFP deletion

@ XhoI digestion.

@ EcoRI digestion.

ARPPa3-EGFP(EcoRI digestion)

0 1 2 3 4 5 6 7 8 9



Transform T=1, 2, 3, 4, 5

16/12

@ PCR screening

using EGFP-1 and ARP2 primers

@ Inoculation

#1 (1.9kb)

#9 (1.5kb)

#11 (1.7kb)

#17 (1.3kb)

#22 (1.1kb)

#31 (0.9kb)

EXHIBIT 8

DEVELOPMENTAL GENETICS 25:158–167 (1999)

Faithful Expression of Green Fluorescent Protein (GFP) in Transgenic Zebrafish Embryos Under Control of Zebrafish Gene Promoters

BENSHENG JU, YANFEI XU, JIANGYAN HE, JI LIAO, TIE YAN, CHOY L. HEW, TOONG JIN LAM, AND ZHIYUAN GONG*

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ABSTRACT Although the zebrafish has become a popular model organism for vertebrate developmental and genetic analyses, its use in transgenic studies still suffers from the scarcity of homologous gene promoters. In the present study, three different zebrafish cDNA clones were isolated and sequenced completely, and their expression patterns were characterized by whole-mount in situ hybridization as well as by Northern blot hybridization. The first clone encodes a type II cytokeratin (CK), which is specifically expressed in skin epithelia in early embryos and prominently expressed in the adult skin tissue. The second clone is muscle specific and encodes a muscle creatine kinase (MCK). The third clone, expressed ubiquitously in all tissues, is derived from an acidic ribosomal phosphoprotein P0 (*arp*) gene. In order to test the fidelity of zebrafish embryos in transgenic expression, the promoters of the three genes were isolated using a rapid linker-mediated PCR approach and subsequently ligated to a modified green fluorescent protein (*gfp*) reporter gene. When the three hybrid GFP constructs were introduced into zebrafish embryos by microinjection, the three promoters were activated faithfully in developing zebrafish embryos. The 2.2-kb ck promoter was sufficient to direct GFP expression in skin epithelia, although a weak expression in muscle was also observed in a few embryos. This pattern of transgenic expression is consistent with the expression pattern of the endogenous cytokeratin gene. The 1.5-kb *mck* promoter/*gfp* was expressed exclusively in skeletal muscles and not elsewhere. By contrast, the 0.8-kb ubiquitous promoter plus the first intron of the *arp* gene were capable of expressing GFP in a variety of tissues, including the skin, muscle, lens, neurons, notochord, and circulating blood cells. Our experiments, therefore, further demonstrated that zebrafish embryos can faithfully express exogenously introduced genes under the control of zebrafish promoters. Dev. Genet. 25:158–167, 1999. © 1999 Wiley-Liss, Inc.

Key words: cytokeratin; muscle creatine kinase; acidic ribosomal phosphoprotein P0; skin-specific; muscle-specific; EGFP

INTRODUCTION

The zebrafish, *Danio rerio*, has emerged as a new model organism for vertebrate developmental biology. As an experimental model, it offers several major advantages, such as easy availability of eggs and embryos, tissue clarity throughout embryogenesis, external development, short generation time, and easy maintenance of both the adult and the young. Recently, systematic mutant screens have generated several hundreds of developmental mutants [Driever *et al.*, 1996; Haffter *et al.*, 1996]. Characterization of these mutants will undoubtedly provide further insights into the mechanisms of vertebrate development. There are two important approaches for understanding the basis of these mutations. One is to isolate the mutant genes for elucidating their function. Currently, several versions of zebrafish genetic maps are available as guides to isolate mutant genes [Postlethwait *et al.*, 1994, 1998; Knapik *et al.*, 1998] and a few mutant genes have been already identified and isolated by positional cloning [e.g., Zhang *et al.* 1998; Brownlie *et al.*, 1998]. The second approach is to demonstrate the function of the mutant genes. This latter approach may require a transgenic technique, for example, to insert a normal gene to rescue a mutant [Yan *et al.*, 1998].

The transgenic approach has become a popular experimental approach for developmental analysis in *Caenorhabditis elegans*, *Drosophila*, and mouse. Usually,

Contract grant sponsor: National University of Singapore.

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Received 4 February 1999; Accepted 19 March 1999

the expression pattern of a transgene reflects that of the endogenous gene from which the promoter for the transgene is derived [for review, see MacDonald and Swift, 1998]. However, in zebrafish, despite the fact that the first transgenic work was reported a decade ago [Stuart *et al.*, 1988], very few transgenic studies have been carried out to address questions on developmental gene regulation. Most of the work on transgenic fish so far used heterologous gene promoters or viral gene promoters [for review, see Hackett, 1993; Gong and Hew, 1995; Iyengar *et al.*, 1996]. As a result, the expression pattern of a transgene in many cases is unpredictable. Recently, using a homologous zebrafish gene promoter, Long *et al.* [1997] have successfully demonstrated the recapitulation of developmental expression of a tissue-specific transcription factor, GATA-1, by introducing a gene construct consisting of the *gata-1* promoter and a reporter gene, *gfp*. The transgenic GFP is specifically expressed in circulating blood cells, reflecting the expression pattern of the endogenous *gata-1*. By a similar approach, Higashijima *et al.* [1997] have also demonstrated the faithful expression of the GFP reporter gene under a zebrafish muscle α-*actin* gene promoter. These experiments indicate the feasibility of using transgenic zebrafish to analyze zebrafish promoters.

With the expressed sequence tag (EST) approach, we have previously identified and isolated more than 400 nonredundant zebrafish cDNA clones from more than 2,000 random cDNA clones that were partially sequenced [Gong *et al.*, 1997; and unpublished data]. These identified clones provide a rich resource for the selection of molecular markers in developmental analyses and for cloning of gene promoters. Based on the expression patterns of homologous genes in other vertebrate species, some of these zebrafish cDNA clones can be inferred to be expressed in a tissue or cell type-specific manner. To achieve rapid isolatation of a gene promoter, we have developed a linker-mediated polymerase chain reaction (PCR) method based on our EST sequences [Liao *et al.*, 1997]. To characterize these gene promoters systematically, the *gfp* reporter gene can be ligated to these promoters and injected into fish embryos at the 1- or 2-cell stage. As the detection of GFP expression is a noninvasive approach, the complete expression pattern can be viewed continuously in live embryos during development.

In the present study, three zebrafish EST clones derived from a cytokeratin gene (*ck*), a muscle creatine kinase gene (*mck*), and an acidic ribosomal phosphoprotein P0 gene (*arp*), were characterized. In situ hybridization and Northern blot hybridization indicate that they were expressed in the skin, in the fast skeletal muscle, and ubiquitously, respectively. Their 5′ flanking regions or promoters were isolated by a linker-mediated PCR approach and ligated with the *gfp* reporter gene. When these zebrafish promoter/*gfp* chimeric constructs were injected into fish embryos, all three transgenic *gfp* constructs were faithfully expressed in early transgenic embryos, indicating the feasibility and fidelity of the transgenic zebrafish system with native zebrafish gene promoters.

MATERIALS AND METHODS

The Zebrafish

Zebrafish were purchased from a local aquarium store and maintained according to the *Zebrafish Book* [Westerfield, 1994].

cDNA Clones

The zebrafish cDNA clones used in the present study were isolated by sequencing randomly selected cDNA clones [Gong *et al.*, 1997], including A39 for cytokeratin, E146 for muscle creatine kinase, and A150 for acidic ribosomal phosphoprotein P0.

In Situ Hybridization

Whole-mount in situ hybridization using digoxigenin (DIG)-labeled riboprobes was carried out as previously described [Korzh *et al.*, 1998]. The plasmid DNAs were linearized with *Pst*I, followed by in vitro transcription reactions with T7 RNA polymerase for the antisense RNA probe. Controls with sense strand probes were also included for in situ hybridization. The embryos were fixed with 4% paraformaldehyde, hybridized with a DIG-labeled RNA probe in a hybridization buffer (50% formamide, 5×SSC, 50 mg/ml tRNA, and 0.1% Tween 20) at 70°C, followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and by staining with the substrates, nitroblue terazolium (NBT) and 5-bromo, 4-chloro, 3-indolil phosphate (BCIP), to produce purple and insoluble precipitates. Some of the stained embryos were embedded in 1.5% agar-sucrose and sectioned using a cryostat (15 μm).

Northern Blot Analysis

Total RNA was isolated from embryos and various adult tissues using Trizol reagent (Life Technologies, USA). A total of 10 μg of RNA was fractionated on 1.2% formaldehyde-agarose gels and transferred to a piece of GeneScreen membrane (DuPont-New England Nuclear). The membrane was hybridized with ^{32}P-labeled cDNA probes at 42°C overnight and washed twice with 0.5% sodium dodecyl sulfate (SDS)/2×SET (0.15 M NaCl; 1 mM EDTA; 20 mM Tris, pH 7.8) and once with 0.1%SDS/0.2×SET at 65°C before autoradiography.

Isolation of Gene Promoters

The promoters were isolated by a linker-mediated PCR method [Liao *et al.*, 1997]. Briefly, zebrafish genomic DNA was digested with selected restriction enzymes individually and modified by T4 DNA polymerase to generate blunt ends if necessary. The digested

genomic DNAs were ligated with a partially double-stranded linker DNA. Two linker-specific primers, L1 and L2, were designed based on the 5′ extended upper strand. Two gene specific primers, G1 and G2, were designed with sequences complementary to the 5′ ends of the respective cDNA clones. To ensure that there is no ATG codon upstream of the reporter gene in the subsequent promoter/*gfp* constructs, the two gene-specific primers or at least the second gene specific primer, G2, was based on the 5′ UTR sequence. Nested PCR was performed with Advantage Tth Polymerase Mix (Clontech). The PCR conditions were as follows: 94°C/1 min, 35 (1st round) or 25 (2nd round) cycles of 94°C/30 s and 68°C/6 min, and finally 68°C/8 min in a Perkin Elmer 480 PCR machine. PCR products were gel purified and cloned into a TA-vector, pT7-Blue (Novagen). The proximal promoter regions were then sequenced for verification based on the 5′ sequences from the cDNA clones.

Construction of Promoter-EGFP Plasmids

The reporter gene vector, pEGFP-1, was purchased from Clontech and it contains a gene encoding a mutant GFP with enhanced fluorescence, resulting from a single amino acid substitution in the fluorescence forming domain and from optimization based on human codon usage preference [Cormack *et al.*, 1996]. Before insertion of the zebrafish promoters, pEGFP-1 was cut with *EcoRI* and *BamHI* at the multiple cloning site. Promoter regions for *ck*, *mck*, and *arp* were amplified by two primers incorporating *Eco*RI and *Bam*HI sites, respectively. The amplified PCR products were cut by *Eco*RI and *Bam*HI, and inserted into pEGFP-1. The resulted chimeric DNAs were named pCK-EGFP, pMCK-EGFP, and pARP-EGFP, respectively.

Microinjection and Detection of GFP Expression

Linearized plasmid DNAs at the concentration of 500 μg/ml (for pCK-EGFP and pMCK-EGFP) in 0.1 M Tris-HCl (pH 7.6)/0.25% phenol red were injected into the cytoplasm of 1- or 2-cell stage embryos. Because of a high mortality rate, pARP-EGFP was injected at a lower concentration (50 μg/ml). Each embryo received 300–500 pl of DNA. The injected embryos were reared in the autoclaved Holtfreter's solution (0.35% NaCl, 0.01% KCl, and 0.01% $CaCl_2$) supplemented with 1 μg/ml of methylene blue. Expression of GFP was observed and photographed under a Zeiss Axiovert 25 fluorescence microscope.

RESULTS

Zebrafish *ck*, *mck*, and *arp* cDNA Clones and Their Expression

To test the feasibility of the zebrafish as a transgenic model for developmental analysis, the skin and muscles were initially targeted for transgenic expression because these tissues are easily identifiable and constitute the greater part of the zebrafish embryo. To complement this study, we also chose a ubiquitous promoter to drive the transgenic expression in all cell types. In order to isolate the zebrafish gene promoters with skin specificity, muscle specificity or ubiquitous feature, three identified zebrafish cDNA clones were chosen from the collection of our tagged cDNA clones or EST clones [Gong *et al.*, 1997]: A39, encoding a cytokeratin (CK) for skin specificity; E146, muscle creatine kinase (MCK) for muscle specificity; and A150, acidic ribosomal phosphoprotein P0 (ARP) for ubiquitous expression. The reason for choosing the *arp* cDNA clone for a ubiquitous promoter is that it is one of the most abundant cDNA clones in both embryonic and whole adult cDNA libraries, on the basis of its frequency among our EST clones [Gong *et al.*, 1997; Gong, 1999].

The three cDNA clones were sequenced completely. A39 is a partial clone, and the complete coding region was recovered by 5′ RACE (rapid amplification of cDNA end). The combined cDNA sequence of the 5′ RACE fragment and the A39 clone is 2,480 nucleotides long, encoding a type II basic cytokeratin (499 amino acids). Its closest homologue in mammals is cytokeratin 8 (65–68% amino acid identity). E146 (1,542 nucleotides) codes for the zebrafish MCK (381 amino acids) and its amino acid sequence shares approximately 87% identity with mammalian MCKs. The amino acid sequence of zebrafish ARP (320 amino acids) deduced from the A150 clone (1,104 nucleotides) is 87–89% identical to those of mammalian ARPs. The complete cDNA sequences have been submitted to Genbank under the access numbers AF134850 (A39 plus the 5′ RACE fragment), AF134851 (E146), and AF134852 (A150).

To demonstrate their expression patterns, whole-mount in situ hybridization was carried out for developing embryos, and Northern blot analysis was carried out for selected adult tissues as well as for developing embryos.

As indicated by whole-mount in situ hybridization, cytokeratin mRNA was specifically expressed in the embryonic surface (Fig. 1A–C) and cross section of in situ hybridized embryos confirmed that the expression was restricted to the skin epithelia (Fig. 1C). Ontogenetically, the cytokeratin mRNA appeared at 3–4 high-power fields (hpf), and it is likely that the transcription of the cytokeratin gene starts at mid-blastula transition when the zygotic genome is activated. By in situ hybridization, the cytokeratin mRNA signal was clearly detected in the highly flattened cells of the superficial layer in blastula and the expression remained in the superficial layer which eventually developed into skin epithelia including yolk sac. In adult tissues, cytokeratin mRNA was detected predominantly in the skin and also weakly in several other tissues including the eye, gill, intestine and muscle, but not in the liver and ovary (Fig. 2A). Therefore, the cytokeratin mRNA is predominantly, if not specifically, expressed in skin cells.

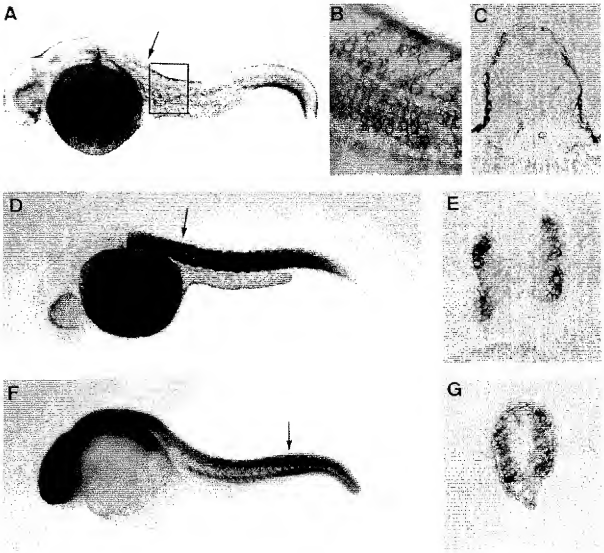

Fig. 1. Expression of *ck* (**A–C**), *mck* (**D,E**), and *arp* (**F,G**) mRNAs in zebrafish embryos as shown by whole-mount in situ hybridization. **A:** A 28-high power field (hpf) embryo hybridized with a *ck* antisense riboprobe. **B:** Enlargement of the midpart of the embryo shown in **A**. **C:** Cross section of the embryo in A. **D:** A 30-hpf embryo hybridized with an *mck* antisense riboprobe. **E:** Cross section of the embryo in **D**. **F:** A 28-hpf embryo hybridized with an *arp* antisense riboprobe. **G:** Cross section of the embryo in **F**. *Arrows*, planes for cross sections; *bar* (**A**), enlarged region shown in **B**.

mck mRNA was first detected in the first few anterior somites in 10 somite stage (14 hpf), at later stages, the expression was specifically in skeletal muscle (Fig. 1D) and in heart (data not shown). When the stained embryos were cross-sectioned, the *mck* mRNA signal was found exclusively in the trunk skeletal muscles (Fig. 1E). In adult tissues, *mck* mRNA was detected exclusively in skeletal muscles (Fig. 2A).

arp mRNA was expressed ubiquitously in adult tissues, although at a low level in the brain (Fig. 2A). In situ hybridization shows intense signals in most tissues, although in the notochord and neural tube, the signal was relatively weak, especially at later stages. An example of a hybridized embryo at 28 hpf is shown in Figure 1F. Under the same conditions, only specific tissues were stained using tissue-specific antisense riboprobes (e.g., Fig. 1A–E); little or no staining was found with a sense riboprobe (data not shown). Thus, the hybridization signals from the *arp* antisense riboprobe was truly derived from *arp* mRNA. These observations confirmed that the *arp* mRNA is expressed ubiquitously.

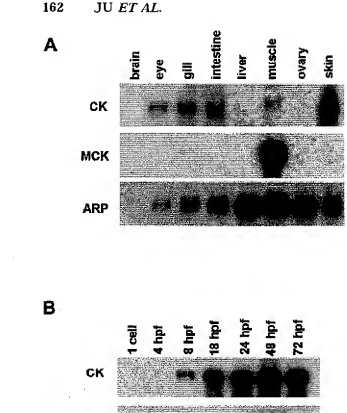

Fig. 2. Northern blot analysis of *ck*, *mck*, and *arp* mRNAs in adult tissues and embryos. **A:** Distribution of *ck*, *mck*, and *arp* mRNAs in adult tissues. Total RNAs were prepared from selected adult tissues as indicated at the top of each lane. **B:** Accumulation of *ck*, *mck*, and *arp* mRNAs in developing embryos from 1 cell stage (before 1 high-power field [hpf]) to 72 hpf, as indicated at the top of each lane. For each panel (**A** or **B**), the same or identical blots were made from the same set of RNAs and hybridized with the *ck*, *mck*, and *arp* probes, respectively.

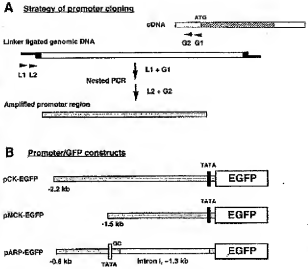

Fig. 3. Schematic representation of strategy of promoter cloning and promoter/*gfp* constructs. **A:** Strategy of promoter cloning. Restriction enzyme digested genomic DNA was ligated with a short linker DNA, and nested polymerase chain reaction (PCR) were performed by primers L1/G1 and L2/G2. L1 and L2 are primers against the linker region. G1 and G2 are gene-specific primers, designed based on a cDNA sequence. **B:** Schematic representation of the three GFP constructs used in the present study. The promoter lengths, TATA boxes, a GC-rich region, and an intron are indicated.

The temporal accumulation of the three mRNAs in developing embryos was also examined by Northern blot hybridization (Fig. 2B). Because Northern analysis is a less sensitive approach, *ck* and *mck* mRNAs were detected later than their detection by in situ hybridization. Both mRNAs were up-regulated after their ontogenetic activation. *arp* mRNA was detected faintly during the first 4 h of development as a short form, which was then replaced by a long form before 8 hpf. Thereafter, the long form *arp* mRNA increased dramatically by 18 hpf and remained constant at ≤72 hpf. Therefore, it is likely that maternal *arp* mRNA (short form) is present before the zygotic genome activation and is replaced by zygotic *arp* mRNA (long form) at mid-blastula transition (approximately 4 hpf).

Generation of *gfp* Transgenic Constructs Using Homologous Zebrafish Gene Promoters

To study the promoter regions controlling tissue-specific expression, three promoters were isolated based on the cDNA sequences by a linker-mediated PCR approach [Liao *et al.*, 1997], as shown diagrammically in Figure 3A. The isolated *cytokeratin* gene promoter is 2.2 kb. In the 3′ proximal region 36 bp upstream of the *ck* cDNA sequence, a putative TATA box perfectly matches the consensus sequence. The 164-bp sequence at one end of the putative promoter fragment are identical to the 5′ UTR of the *cytokeratin* cDNA. Thus, the isolated fragment was indeed derived from the same gene as the *cytokeratin* cDNA clone. Similarly, a 1.5-kb 5′ flanking region was isolated from the muscle *creatine kinase* gene, with a putative TATA box in its 3′ proximal region (35 bp from the start of the cDNA sequence). The 3′ region is also identical to the 5′ portion of the *mck* cDNA clone. A 2.1-kb fragment was amplified from the *arp* gene. By alignment of its sequence with the *arp* cDNA, we found a 1.3-kb intron in the 5′ UTR. As a result, the isolated ARP promoter is only about 0.8 kb long. Although there is a putative TATA box located 170 bp from the start of the cDNA sequence, there is a GC-rich region immediately before

the cDNA sequence. Therefore, the *arp* promoter may be a GC-type ubiquitous promoter. All the three promoter fragments were inserted to the *gfp* reporter gene vector, pEGFP-1, and designated as pCK-EGFP, pMCK-EGFP, and pARP-EGFP, respectively (Fig. 3B).

Transgenic Expression of GFP Under a Skin-Prominent Promoter

When pCK-EGFP was injected into 1- or 2-cell stage embryos, GFP expression started at about 4 h after injection, which corresponded to the stage of approximately 30% epiboly. About 55% of the injected embryos expressed GFP at this stage. The early expression was always in the superficial layer of cells, mimicking endogenous expression of the *ck* gene as observed by in situ hybridization (Fig. 4A). However, the transgenic expression was mosaic as it was observed only in some but not in all surface cells. At 24 h after injection, the strongest GFP expression was usually found in the yolk sac, especially at the boundary between the yolk and the embryo. GFP expression appeared only in the epithelial cells on the embryonic surface (Fig. 4B–D). By 48 hpf, about 57% of surviving embryos expressed GFP and 100% of GFP-expressing embryos showed expression in skin epithelial cells. In about 8% of surviving embryos, a weak GFP expression was also found in muscle cells, but not in the central nervous system (CNS), notochord, lens, and other tissues that frequently expressed GFP under the ubiquitous promoter from the *arp* gene (see below). The summary of injected embryos and the patterns of GFP expression are shown in Table 1.

GFP expression from pCK-EGFP was still visible when the larvae were 1.5 months old. About 20% of the fish initially expressing GFP remained GFP-positive, and the overall expression level decreased. Skin-specific expression at this stage was found mostly in the head region and the fins. However, expression in the muscle cells showed no obvious decrease of the fluorescence intensity.

Transgenic Expression of GFP Under a Muscle-Specific Promoter

Under the *mck* promoter, no GFP expression was observed in early embryos before muscle cells became differentiated. By 24 hpf, about 12% of surviving embryos expressed GFP strongly in muscle cells and these GFP-positive embryos remain GFP positive at the hatching stage (48 hpf) (Table 1). The GFP expression was always found in many bundles of muscle fibers, mainly in the mid-trunk region (Fig. 4E), and no expression was ever found in other types of cells. For the first 2 weeks of development, there was no decrease in fluorescence intensity, further indicating that the transgenic GFP expression in muscle cells can last longer. When examined 1.5 months after injection, the fluorescent muscle fibers were still visible.

Although the endogenous *mck* gene was also expressed in the heart, expression of exogenously introduced pMCK-EGFP construct was not detected in the heart. This might indicate that the promoter region used does not include the *cis*-element for heart expression. Alternatively, or more likely, the expression in the heart could rarely occur because heart tissues constitute only a small portion of the embryo, and the probability of segregating the transgene into the heart lineage might be too low due to the mosaic segregation [Westerfield *et al.*, 1992]. Consistent with this, among the 244 embryos injected with the ubiquitous promoter construct, pARP-EGFP, none showed GFP expression in the heart. Thus, it will be interesting to examine heart expression of *mck-gfp* in F1 generation from germline transgenic zebrafish.

Transgenic Expression of GFP Under a Ubiquitous Promoter

Expression of *arp-egfp* was first observed 4 h after injection at the 30% epiboly stage. The timing of expression was similar to that of pCK-EGFP injected embryos. However, unlike the *ck-egfp* transgenic embryos, GFP expression under the *arp* promoter occurred not only in the superficial layer of cells but also in deep layers of cells (Fig. 4F). In some batches of injected embryos, almost 100% of the injected embryos expressed GFP initially. At later stages, when some embryonic cells become differentiated, GFP expression was detected frequently in the skin, muscle, lens, neural tissues, notochord, and circulating blood cells (Fig. 4G–K). In early embryos of ≤24 hpf, GFP expression was detectable mostly in neural cells and skin epithelia (Fig. 4G,H) and after 48 hpf neural GFP expression was extinguished in most GFP-expressing embryos, although the epithelial and muscular expression continued for more than 1.5 month. The disappearance of GFP in neural cells is consistent with the observation of a low level of *arp* mRNA expression in CNS during late stages of embryonic development (Fig. 1G) and in the adult brain (Fig. 2A). For muscle expression, unlike the GFP expression in pMCK-EGFP injected embryos, the GFP expression with pARP-EGFP occurred only in scattered muscle fibers. In addition, the intensity of GFP fluorescence in this case was much weaker than that observed in pMCK-EGFP-injected embryos.

For some unknown reasons, the embryos injected with pARP-EGFP showed a poor survival rate. By 48 hpf, only 23% of the injected embryos survived, while the survival rates were 44% and 53% for the embryos of the same stage injected with pCK-EGFP and pMCK-EGFP, respectively (Table 1). For those pARP-EGFP-injected embryos that survived at 48 hpf and expressed GFP, most of them had obvious developmental abnormalities, such as shortening of trunk and loss of brain or tail. A similar low survival rate and developmental abnormalities were also observed in embryos injected

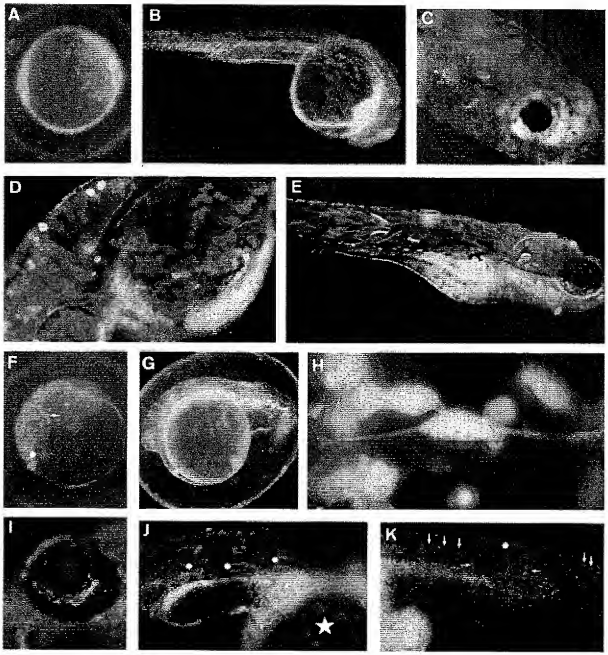

Fig. 4. GFP expression in transient transgenic zebrafish embryos. Embryos were injected with pCK-EGFP (**A–D**), pMCK-EGFP (**E**), or pARP-EGFP (**F–K**). **A:** Expression of GFP at 6 high-power fields (hpf). Note that the GFP expression is only in the flattened surface cells. **B:** GFP expression in a 24-hpf embryo. **C:** GFP expression in the head region of a 96-hpf embryo. **D:** GFP expression in a 48-hpf embryo. The picture focuses on skin cells. **E:** Muscle-specific expression of GFP under the *mck* promoter in a 72-hpf embryo. **F:** GFP expression at 6 hpf. Note that GFP expression is detected both in surface cells (*arrow*) and in the deep layer cells (*asterisk*, out of focus). **G:** GFP expression in a 22 hpf embryo and most expression was detected in developing nervous system at this stage. **H:** High magnification of a 22-hpf embryo showing GFP expression in individual neurons. **I:** GFP expression in lens. **J,K:** GFP expression in two individual embryos (**J**, 20 hpf; **K**, 24 hpf). *Asterisks*, muscle fibers; *star*, skin cells; *vertical arrows*, neurons; *horizontal arrows*, notochord.

TABLE 1. Summary of *gfp* Transgenic Zebrafish Embryos

Stages/ constructs	1–2 cells Injected	50% epiboly (4 hpf) Survival (%)	50% epiboly (4 hpf) Expression (%)	Hatching (48 hpf) Survival (%)	Hatching (48 hpf) Expression (%)	Tissue distribution of GFP at 48 hpf[a] Skin	Muscle	CNS	Others[b]
pCK-EGFP	440	230 (52)	102 (44)	193 (44)	111 (57)	111	16	0	0
pMCK-EGFP	226	160 (71)	0 (0)	121 (54)	14 (12)	0	14	0	0
pARP-EGFP	244	159 (62)	104 (65)	57 (23)	37 (65)[b]	37	14	16	18

[a]The combination of these expressing individuals exceeds the number of the hatched-out larvae as one larvae may express GFP in several tissues. Skin, including yolk sac and fins; CNS, central nervous system; other, including lens, blood cells, and notochord.
[b]Only 15 embryos showed normal development.

with another ubiquitously expressing EGFP construct, pCMV-EGFP, which has a strong cytomegaloviral (*cmv*) promoter (data not shown). Thus, ubiquitous expression of EGFP might be detrimental to embryonic development probably because a strong GFP expression could interfere with the normal cellular function of certain cells, adversely affecting the embryonic survival. Consistent with this, embryos showing high GFP expression at early stages usually died early or had more severe abnormalities. By contrast, the strong GFP expression in the skin and in the skeletal muscle under the *ck* and *mck* promoters, respectively, have no such adverse effect on embryonic survival, suggesting that the skin and muscle cells might tolerate a high level of transgenic GFP expression.

DISCUSSION

A critical factor for successful transgenic research is the design of a DNA construct, which consists of a gene promoter, a structural gene, and a transcription termination signal. Among the three components, promoter is most important in directing the structural gene to be activated at a correct stage and in a proper tissue. However, because of the lack of zebrafish gene promoters, most of the early work on transgenic zebrafish used heterologous promoters from viruses or from other species of animals [e.g., Stuart *et al.*, 1988, 1990; Westerfield, 1992; Lin *et al.*, 1994, Amsterdam *et al.*, 1995; Muller *et al.*, 1997]. Thus, until recently, it was unclear whether transgenic zebrafish is capable of expressing the transgene faithfully. Recently, two groups have successfully reported the faithful expression of transgenes using zebrafish gene promoters [Meng *et al.*, 1997; Long *et al.*, 1997; Higashijima *et al.*, 1997]. In the present study, we have demonstrated the faithful expression of a reporter gene under three zebrafish gene promoters of different tissue specificity by a transient transgenic expression assay. Thus, we further confirmed the validity of zebrafish as a transgenic system to analyze tissue specific gene expression and to test the function of a gene.

The first zebrafish gene promoter we used is a skin-specific (or -prominent) promoter derived from a *cytokeratin* gene, which is expressed predominantly in skin epithelial cells. Consistent with the expression pattern of the endogenous *cytokeratin* gene, its promoter directed the *gfp* transgene expression predominantly in skin cells. In only about 8% of embryos, a weak GFP expression was also observed in muscle cells. This may be explained by the fact that the *cytokeratin* gene is also weakly expressed in the muscle tissue as indicated by Northern blot hybridization (Fig. 2A). The second zebrafish promoter used was derived from a muscle *creatine kinase* gene. As predicted, the GFP expression under the muscle specific *mck* promoter was exclusively in skeletal muscle cells and no GFP expression was found in any nonmuscle cell. The ubiquitous promoter from the *arp* gene directed the transgenic GFP expression in many different types of cells, such as skin epithelia, neural cells, notochord, lens, blood cells and muscle, again consistent with the expression of the endogenous *arp* gene.

The temporal activation of the three gene promoters is also in good agreement with the timing of the activation of these endogenous genes during normal development. Both *ck* and *arp* mRNAs can be detected as early as the blastula stage (before 4 hpf, data not shown) and it is likely that these genes are activated when the zygotic genome is activated at mid-blastula transition. Consistent with this, the GFP expression under the *ck* and *arp* promoters was observed quite early, at about 4 h after injection (approximately 30% epiboly stage or approximately 5 hpf), which is shortly after the mid-blastula transition. By contrast, the GFP expression under the *mck* promoter was only observed after muscle has formed.

It is worth mentioning that, in all cases, the transient transgenic expression is mosaic and highly variable among the embryos injected with the same DNA construct; i.e., not all skin cells or muscle cells expressed the *gfp* transgene, and not every embryo showed identical pattern of expression. These phenomena are primarily attributable to the differential segregation of the injected DNA during embryogenesis, as documented in early transgenic fish research [for review, see Hackett, 1993; Gong and Hew, 1995; Iyengar *et al.*, 1996]. Nevertheless, the transient transgenic system remains an effective and reliable system to investigate the

pattern of gene expression by analysis of a large number of individuals [Westerfield *et al.*, 1992, Meng *et al.*, 1997; Muller *et al.*, 1997]. It also provides a rapid, convenient assay with which to dissect the *cis*-elements controlling the temporal and spatial patterns of expression. For example, Westerfield *et al.* [1992] have demonstrated the expression domain of a mouse homeobox gene in transgenic zebrafish by analysing a large number of embryos injected with a mouse homeobox gene promoter with a *LacZ* reporter gene. More recently, Meng *et al.* [1997] used the transient transgenic zebrafish system for successful dissection of the *cis*-elements of a transcription factor gene, *gata-2*, responsible for hematopoietic, enveloping layer and neuronal expression. In the present study, a 1.5-kb 5' flanking sequence from the *mck* gene successfully directed the reporter gene to be expressed specifically in the skeletal muscle, indicating that the cis-elements for skeletal muscle expression are located within the 1.5-kb region. Deletion analysis of the 1.5-kb promoter region will further map the region for skeletal muscle specificity and site-directed mutagenesis can be used to further confirm the mapped *cis*-elements. Similarly, the region determining the skin specificity must be located within the 2.2-kb upstream region of the cytokeratin gene.

An alternative transgenic approach to analyze gene regulation is to develop germline transgenic zebrafish. The transgenic expression pattern would likely be reproducibly observed in F1 and subsequent generations [Stuart *et al.*, 1990; Long *et al.*, 1997; Higashijima *et al.*, 1997]. This approach may eliminate the problem of mosaic expression and, in most cases, the transgenic lines will have correct patterns of expression, mimicking those of endogenous genes from which the promoters derived from. However, variable transgenic expression could still occur among different transgenic lines, attributed to a chromosomal effect [Stuart *et al.*, 1990]. To overcome the latter problem, efforts have been made in transgenic mice by using certain chromosomal controlling elements, such as locus control region from the globin gene cluster [Grosveld *et al.*, 1987] and matrix attachment region from the chicken lysozyme gene [McKnight *et al.*, 1992]. In both cases, a more consistent expression of transgene among different transgenic lines was observed. However, such elements have not been tested in the zebrafish system.

The development of stable *gfp* transgenic lines should also be valuable for many other studies. Since detection of GFP is a noninvasive approach, expression of GFP can be continuously observed by epifluorescence microscopy. By selecting a tissue-specific promoter, the developmental expression pattern of the gene from which the promoter is derived can be recapitulated [Long *et al.*, 1997]. With the availability of a wide range of zebrafish cDNA clones [Gong *et al.*, 1997; Gong, 1998] and the ability to isolate zebrafish gene promoters rapidly [Liao *et al.*, 1997], many developmental processes could be recapitulated in this way. Furthermore, *gfp* transgenic lines will also facilitate the studies of cell lineage and cell migration if GFP is expressed in a tissue-specific manner. *gfp* transgenic zebrafish, particularly under a ubiquitous promoter, will also be valuable for cell transplantation and nuclear transplantation experiments because the GFP and the *gfp* transgene can be conveniently used as cellular and genetic markers.

The zebrafish has become an increasingly popular model for vertebrate developmental analysis. Although the use of zebrafish as an experimental model has many advantages, there are also several drawbacks. One drawback is the relatively low number of the cloned genes as compared with other model organisms. This problem has been partially alleviated by a massive cloning strategy of using an EST approach [Gong *et al.*, 1997; Gong, 1998]. Another drawback is the lack of in vitro cell lines derived from the zebrafish. To overcome the second problem, a transgenic approach will be useful. For example, using a tissue- or cell type-specific promoter to drive an oncogene, tissue-specific tumor could be developed from transgenic zebrafish, and immortalized zebrafish cell lines may be established in this way, as demonstrated in transgenic mice [Efrat *et al.*, 1988]. Thus, characterization of more tissue specific zebrafish gene promoters is important and valuable at this stage of zebrafish developmental biology.

ACKNOWLEDGMENTS

We thank Dr. C.H. Tan for proofreafing of the manuscript, and Drs. V. Korzh and L. Orban for their expert comments and criticisms. Y.X. and J.L. are supported by NUS postgraduate scholarships.

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EXHIBIT 9

8/6/98.

Total RNA isolation by TRIzol.

in 20 ml DEPC H2O

(200 X dilution)

	A_{260}	A_{280}	A_{260}/A_{280}	[RNA] (μg/ml)
2hr. embryo	0.421	0.279	1.565	3.448
6hr "	0.403	0.237	1.7026	3.224
8hr "	0.502	0.295	1.7046	4.016
12hr "	0.588	0.368	1.6011	4.712
16hr "	0.299	0.249	1.4609	2.392
16 hr "	0.528	0.343	1.5382	4.224
18hr "	0.440	0.289	1.5240	3.52
20hr "	0.637	0.402	1.5843	5.096
24hr "	0.618	0.397	1.5566	4.944
48hr "	0.771	0.478	1.5420	6.168
zero stage Adult	0.984	0.571	1.7245	7.872

Notes:

RNA extraction

① take embryos from different stages (100~200), add 100 ml TRIzol, homogenize the embryos.

② add 900 ml of TRIzol, homogenize further

③ Incubate at RT for 5 min

④ add 200 ml chloroform, vortex at top speed for 30 sec, incubate at RT for 2-3 min.

⑤ 12000 g x 15', at 4°C

⑥ Transfer the clear aqueous phase (about 500~900 ml), add 500 ml Isopropanol mix.

⑦ Incubate at RT for 10 min

⑧ 12000 g x15', at 4°C

⑨ wash with 1 ml 70% ethanol. 7500 g x 5' at 4°C

⑩ Air dry

⑪ redissolve in 20 ml DEPC treated water.

10/6/98.

Running RNA gel

RNA samples:

	for 1 sample	for 17 samples	
formamide	10 ml	170 ml	32.6 ml x 8 tubes
37% formaldehyde	4 ml	68 ml	
10 x Mops	2 ml	34 ml	
EB	1.3 ml	5 ml	
RNA	10 ug (7.7 ml)		
	/20ml.		

	8 hr	12 hr	14 hr	16 hr	18 hr	20 hr	24 hr	48 hr
[RNA] (ug/ml)	4.016	4.712	2.392	4.226	7.52	5.096	4.948	6.168
for 1 sample (uL)	2.5	2.12	4.18	2.37	2.84	1.96	2.02	1.62
2 samples (uL)	5	4.24	8.36	4.74	5.68	3.92	4.04	3.24
H_2O (ml)	2.4	2.16	/	2.66	1.72	3.48	3.36	4.16

Heat the sample at 68°C for 10 min. cool on ice

Add 3 ml of loading dye to each tube.

Load 21.5 ml of RNA sample. (for 14hr embryo RNA, load 22 ml / lane)

Making gel (100 ml)

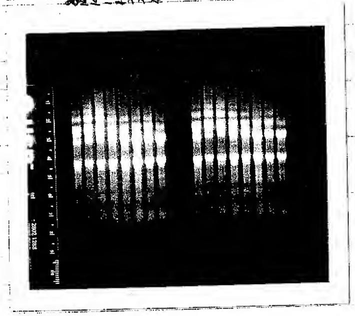
Agarose 1.2 g

10 x Mops 10 ml

H_2O 73 ml

Heated, cooled to 65°C, add 17 ml 37% formaldehyde.

Running buffer (700 ml)	600 ml
10 x Mops 70 ml	60 ml
37% formaldehyde 56 ml	48 ml



Northern blot.

11/06/98.

① PCR amplify fagment.

for 1 sample

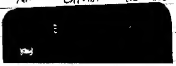
10×PCR buffer	5	94°C 5'
2mM dNTP	2.5	94°C 30" }
$MgCl_2$	3	57°C 1' } 35 cycles
T7	0.5	72°C 1' }
SK	0.5	72°C 5'
Taq	0.2	
Template	1	
H_2O	37.7	
	/50 ul	

M E68 E45 E371 A84 MCK E134 MLC2 A228

② Purify the fagment by QIAgen into 30 ul H_2O each

run 2 ul on agarose gel

(240ng)
λHindIII E371 A84 MLC A228



12/06/98.

Summary of in situ hybridization results:

	19 hpf (20 somites)	fin bud	heart	Notes
E371	20 somites	✓	x	strong signal at 14hpf
E146	18 "	✓	✓	
E68	~18 "	✓	x	
E134	<14 "	✓	x	
E405	<14 "	faint	x	
A228	19 "	✓	x	strong signal at 16hpf
A354	20 "	✓	x	
E72 (MLC2)	14 "	✓	x	

Desmin — strong signal at 12hpf

The expression sequence of muscle genes

Desmin

↓

E371 (α-tropomyosin) A354 (troponin C)

↓

A228 (tropomyosin fast muscle)

↓

E146 (MCK)

↓

E68 (β- myosin heavy chain)

↓

MLC2 fast muscle

↓

E134 (troponin T) E405 (parvalbumin)

13/06/98.

Random primer Labelling (E371, MLC)

DNA fragment	1 ul
dCTP	1 ul
dGTP	1 ul
dTTP	1 ul
^{32}P- dATP	2.3 ul
Random primer Mix	7.5 ul
H_2O	10.5 ul
	24.5 ul Mix gently

add Klenow fragment 0.5 ul

25 ul Mix.

RT incubation for 70 min.

Add 2.5 ul stop buffer.

Column filtration (Nick column, equilibrate by 4ml TE. add the reaction mix wash with 400 ul TE x 3 times)

Count (2 ul + 3 ml BCS.)

		cpm	cpm / 1 ul	5ml Hyb. Buf. need	Actually add
E371	①	3139.5			
	②	77523.1	38761.5	129 ul	130 ul
	③	19013.9			
MLC	①	2255.1			
	②	77513.0	38756.5	129 ul	130 ul
	③	13792.2			

Prehybridization at 42°c for 2hrs (add 5 ml Hyb. buf for each membrane)

Denature the probe at 95°c for 3 min. cool on ice,

change 5ml fresh Hyb. buf. with 130 ul probe.

Incubate at 42°c overnight

E371 — #① membrane

MLC — #② membrane

H16198.

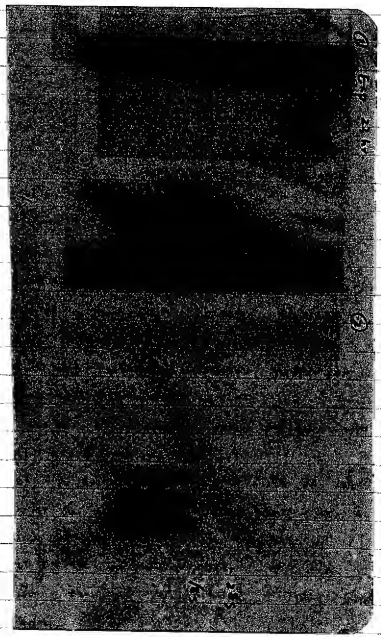
Washing.

① ~~2×SET~~ 2×SET / 0.1% SDS , RT 20', 2 changes

② 2×SET / 0.1% SDS , 65°C 30' 2 changes

③ 0.2×SET / 0.1% SDS 65°C 10' (signal ≤ 20 cpm)

Autoradiography (-80°C)



16.6.98

Re-run RNA gel

① Making gel 100 ml (1.2%)

Agarose 1.2 g
10×MOPS 10 ml
H₂O 73 ml
→ melt → cool to 65°C
add 17 ml 37% formaldehyde

② Running buffer (600 ml)

10× MOPS 60 ml
37% formaldehyde 48 ml
H₂O 492 ml

③ RNA sample

for one sample (20 ml)

formamide 10 µl
37% formaldehyde 4 µl
10×MOPS 2 µl
RNA 10 µg (2.9 µl)
EB 0.1 µl

(RNA)

	8 hr	12 hr	14 hr	16 hr	18 hr	20 hr	24 hr	48 hr	Adult
RNA (µl)	2.5	2.12	≤	2.5	2.86	1.96	2.02	1.62	1.12
H₂O (µl)	1.4	1.78	–	1.53	1.06	1.94	1.98	2.28	2.63

④ Heat the RNA samples at 65°C for 10', cool on ice

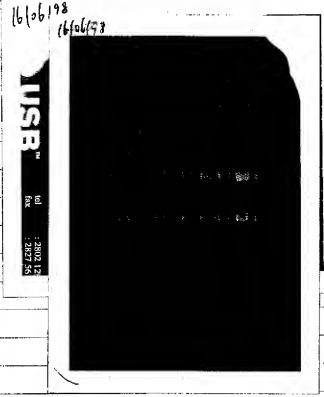
Load the samples with 2 µl loading buffer.

Run at 70 V.

⑤ Transfer to membrane

16/06/98

16/06/98



← 28S 3.3kb

← 18S 1.8kb

23/06/98.

RNA extraction.

1. Embryos of different stages (200 each) → thaw at RT, quickly add 150 ml of TRIzol. homogenize.
2. Add 850 ml of TRIzol, homogenize further.
3. Incubate at RT for 5 min
4. Add 200 ml of chloroform, shake by hand for 15 seconds. Incubate at RT for 3 min.
5. 12000 g x 15', at 4°c.
6. Transfer the upper clear aqueous phase (700 - 900 ml) add equal volumn of isopropanol mix, incubate at RT for 10 min
7. 12000 g x 10', at 4°C.
8. Wash with 1 ml 70% ethanol 7500 g x 5' at 4°c.
9. Air dry.
10. Redissolve into DEPC-treated water (15 ml - 40 ml)
11. 60°C incubate for 10 min

check RNA concentration

(200× dilution)

	total volume	A_1	A_2	A_1/A_2	[RNA] (ng/ml)
10h	20 ml	0423	0.245	1.7284	3.384
12h	25 ml	0.368	0.218	1.6836	2.944
14h	30 ml	0.358	0.208	1.7159	2.864
16h	40 ml	0.376	0.215	1.7513	3.008
17h	15 ml	0.430	0.248	1.7317	3.44
18h	30 ml	1.465	0.275	1.6924	3.72
20h	40 ml	0.409	0.241	1.6902	3.272
24h	20 ml	0.731	0.422	1.7312	5.848
48h	25 ml	0.776	0.452	1.7160	6.208
72h	15 ml	1.088	0.629	1.7304	8.704

30/06/98

Run RNA gel

RNA samples.

	for 1 sample (18ml)	for 12 samples	
formamide	9 ml	108 ml	14.5 ml × 11 tubes
37% formaldehyde	3.6 ml	43.2 ml	
10× MOPS	1.8 ml	21.6 ml	
EB	0.1 ml	1.0 ml	
RNA (10 ng)	3.5 ml		

	10h	12h	14h	16h	17h	18h	20h	24h	48h	72h	Adult
[RNA] (ng/ml)	3.384	2.944	2.864	3.008	3.44	3.72	3.272	5.848	6.208	8.704	7.872
RNA (ml)	2.96	3.40	3.5	3.32	2.91	2.69	3.06	1.71	1.61	1.15	1.27
H₂O (ml)	0.54	0.1	/	0.18	0.59	0.81	0.44	1.79	1.89	2.35	2.23

Heat at 65°C for 15'. cool on ice

7/7/98.

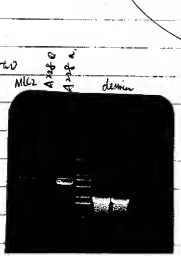
1. PCR amplify desmin fragment.

10×PCR buffer	3	94°C 5'
2mM dNTP	1.5	92°C 30"
$MgCl_2$	1.8	58°C 1' } 35 cycles
T_7	0.3	72°C 1'
SK	0.3	72°C 5'
Taq	0.1	
Template	1	purify the fragment into 30 ml H_2O
H_2O	22 / 30 ml	[DNA] is about 10 ng/ml

2. A228 and MLC2 RNA probe synthesis

MLC2 (2mg/ml)	2 ml	A228 Q 10 ml + 4ml H_2O
10×Buffer	2 ml	A228 A 10 ml
10×BSA	2 ml	
BamHI	2 ml	
H_2O	12 ml	

37°C water incubation for 2 hr.



4/7/98

Run RNA gel, make another RNA blot.

△Note!

There is a leakage below 18S rRNA!

This blot can only be used for β signal > 18 rRNA, eg. β-actin

14/7

32P-probe labelling

MLC2	1 ml	+	12.5 ml H_2O
β-actin	4 ml	+	7.5 ml H_2O

↓

95℃ incubate 5', cool on ice.

↓

Add the following

dCTP	1 ml
dTTP	1 ml
dGTP	1 ml
Random primer MIX	7.5 ml
32P-dATP	2.5
Klenow	0.5 ml

RT incubation 70 min.

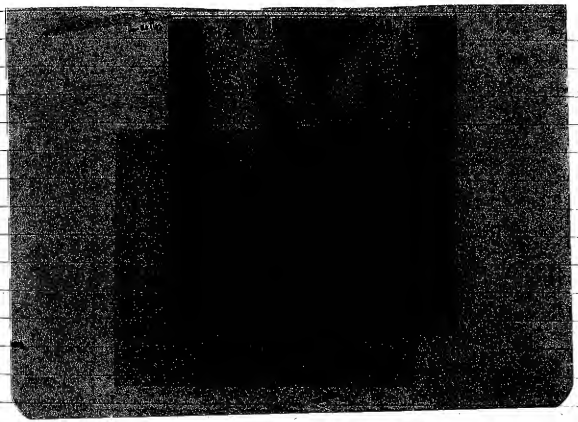
↓

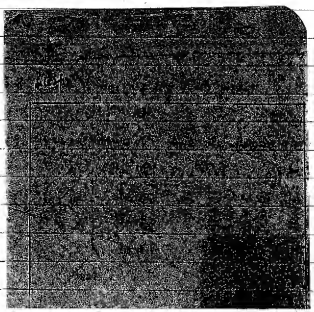
Add stop buffer 2.5 ml.

Scintiling counting

	cpm	cpm / ml of probe	5 ml of hyb. buf. need
NHC2	2939064	1.46×10^5	34.2 ml
β-actin	2864382	1.42×10^5	35.2 ml

45°C hybridize O/N.





22/07/98.

RNA extraction.

200x dilution to check concentration

JY's) 8hr	0.193	0.128	1.5024	1.544 ug/ml	
12hr	0.523	0.332	1.5752	4.184	15 ul
14hr	0.642	0.414	1.5521	5.136	15 ul
16hr	0.410	0.274	1.4980	3.28	20 ul
17hr	0.567	0.369	1.5376	4.536	15 ul
(JY's) 18hr	0.293	0.199	1.4715	2.344	
20hr	0.558	0.362	1.5435	4.464	20 ul
24h	0.681	0.433	1.5724	5.448	20 ul
48h	0.816	0.529	1.5412	6.528	15 ul
72h	1.004	0.635	1.5819	8.032	15 ul

23/07/98.

Run RNA gel.

	8h	12h	14h	16h	18h	20h	24h	48h	72h	A
[RNA]	1.544	4.184	5.136	3.28	2.344	4.464	5.448	6.528	8.032	7.872
10 ug need	6.47 (4.0)	2.39	1.95	3.05	4.26 (3.9)	2.24	1.84	1.53	1.25	1.27
20 ug		4.78	3.9	6.1	~~7.75~~ 3.5 7.0	4.48	3.68	3.06	2.5	2.54
~~H2O~~		~~3.02~~	~~3.9~~	~~1.7~~	/	~~3.32~~	~~4.12~~	~~4.74~~	~~5.3~~	~~5.26~~
H2O		2.22	3.1	0.9	/	2.52	3.32	3.94	4.5	4.46

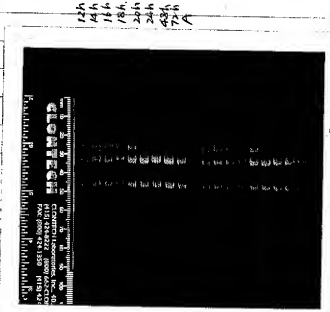
RNA samples

	for 1 (20 ul)	~~for 19 samples~~	
formamide	10 ul	~~190 ul~~	~~16.1 ul x 18 tubes~~
37% formaldehyde	4 ul	~~76 ul~~	
10x MOPS	2 ul	~~38 ul~~	
EB	0.1 ul	~~1.9 ul~~	
RNA <	3.9 ul		

RNA samples

	for 1 ml (1/8 ml)	for 19 samples
formamide	9 ml	~~17 ml~~ 171 ml
37% formaldehyde	3.6 ml	~~6~~8.4 ml
10 x MOPS	1.8 ml	~~3~~4.2 ml
FB	0.1 ml	1.8 ml
RNA	3.5 ml	

} 29 ml × 9 tubes



24/07/98

1. Making A228. E134. A485 β dig-RNA probes

① Digestion

Plasmid (A228, E134)	10 ml
BamHI	1 ml
10 x Buffer	2 ml
10 x BSA	2 ml
H_2O	5 ml / 20 ml

37°C incubation for 1.5 hr.

Run 1ml on agarose gel for checking

③ probe synthesis

E4b5, A228 ① E04 ② + H_2O — 11 ul
(4ul) (9.5ul)

5× transcription buffer — 4 ul
10× dig-NTP — 2 ul
DTT — 1 ul
RNAsin — 1 ul
T7 poly. (RNA) — 1 ul
/ 20 ul.

37°C incubation for 2 hr.

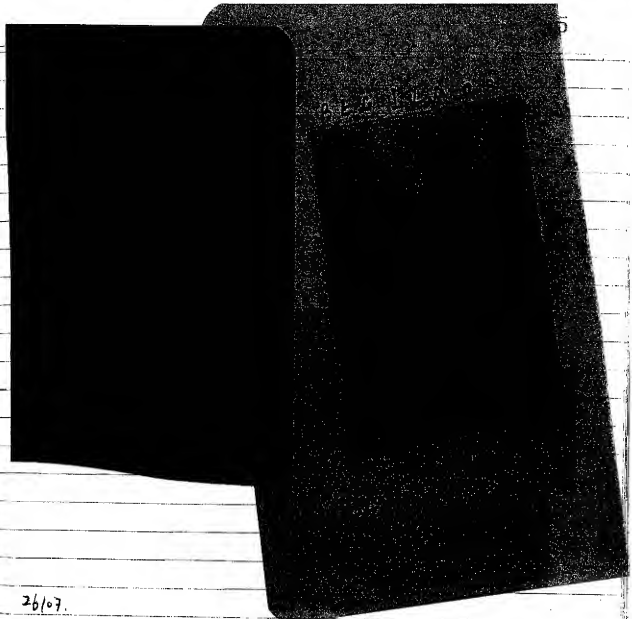
Add 1 ul EDTA (0.5M), 2.5 ul LiCl (4M), 75 EtOH 100%
~~20%~~ -20°C incubation overnight.

④. Northern Hybridization.
X1 — Mlc 2.
X2 — β-actin.

	cpm / 2 ul	cpm / ul	5 ml need
Mlc 2	133422.1	0.67×10^5	74.6
β-actin	92356.3	0.46×10^5.	108.7

According to ~~32P~~ ^{32}P decay date table (10 day)
the remain radioactivity should be 0.6 times of original one

	original cpm / ul	present cpm / ul	5 ml need	Actually add
Mlc 2	1.46×10^5	0.87×10^5	57.5	60 ul
β-actin	1.42×10^5	0.85×10^5	58.8	~~65~~ 70 ul



26/07.

① test the probe A228. E465. A137 by in situ hybridization
using 5 20hrs embryo for each probe (3μl in 300μl Hyb. buf.)

② Resuspend 18th tRNA.
dissolve in 20 μl DEPC H2O
Measure the concentration (200 x dilution)

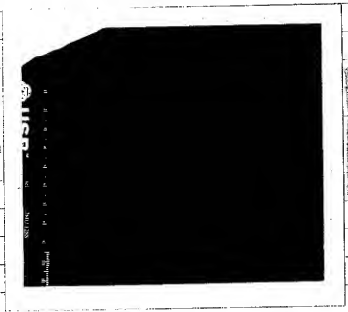
A260	0.377
A280	0.375
A1/A2	1.[illegible]0
[RNA]	4.616 μg/μl
10μg need	2.17 μl

28/07

Run RNA gel.

RNA samples (3.5 ml each

	12 h	14h	16h	18h	20h	24h	48h	72h	18
10 ng need	2.39	1.95	3.05	2.17	0.44	1.34	1.53	1.25	2.27
20 ng need	4.78	3.9	6.1	4.34	448	2.68	3.06	2.50	2.54
H2O	2.22	3.1	0.9	2.66	2.52	3.32	3.94	4.5	4.46



* Transfer to membrane

29/07

Hybridization with Nick and RRP probes

	cpm/ml		5×10^6 need	
Nick	1.1×10^5 × 0.6	= 0.66×10^5	75.8 ml	(76 ml)
RRP	1.3×10^5 × 0.6	= 0.78×10^5	64.1 ml	(64 ml)

3/18/98

Random Primer Probe Labelling

desmin, E465 fragment 1 ml

H_2O 10.5 ml

↓

95°C, 5', cool on ice.

↓

dCTP 1 ml. dTTP 1 ml dGTP 1 ml

α-dATP 2.5 ml

Random Primer 7.5 ml

Klenow 0.5 ml

↓

RT for 1h ~~min~~.

	P32_CPM	RPos	CTime	1 ml.	5ml need
desmin	477569.0	1	120		
E465	310105.9	2	120	2.4×10^5 cpm	20.8 ml
	520658.0	3	120		
	292208.4	4	120	1.55×10^5 cpm	32.3 ml

11/03/98

Re-do Northern hybridization of desmin.

use ~~membrane~~ membrane ②

check cpm.

cpm / 5 ml	cpm / ml.	5 ml Hyb. buf. need	~~actually add~~
321869.2	1.6×10^5	31.3 ml	~~77 ml~~

13/08/93.

1. Random labelling E134 and E371

DNA (E134 / E371)	1 ml
H_2O	10.5 ml

95°C for 5', cool on ice

Add the following

dATP	1 ml
dGTP	1 ml
dGTP	1 ml
^{32}P dCTP	2.5 ml
Random Primer	7.5 ml
Klenow	0.5 ml

RT for 1 hr.

2. Check cpm.

	cpm / 2 ml	cpm / ml	5ml Hyb. Buf. need
E134	1596.74.1	0.8×10^5	62.5 ml
E371	2015.0.6	$\cdot 10^5$	50 ml.

3. Hybridization at 65°C for overnight.

A-355 E68 ⑧ 98.8.20
24hr.

E271 ⑧ 98.8.15 24hr. 室温
12h
16h
18h
20h
24h
48h
72h
A
E136 ⑧ 98.8.15 24hr. 室温
2h
4h
6h
8h
20h
24h
48h
72h
A

12/08/98

Automated sequencing reaction the cDNA clones

① check plasmid concentration

run 1 ml on agarose gel.

② sequencing reaction

plasmid		Big dye	T_7 / SK	H_2O
E134 (1.0Kb)	1 ml	7	1 / 1	11
A254 (6.9Kb)	2 ml	7	1 / 1	10
A228 (1.0Kb)	1 ml	7	1 / 1	11
E405 (0.7Kb)	2 ml	8	1	9
E371 (1.4Kb)	1 ml	8	1 / 1	10
E68 (1.3Kb)	4 ml	8	1 / 1	7
ARPP(2.1Kb)-PT_7	1ml	8	M13 1	10

③ PCR (96w)

96°C 10"
50°C 5" } 25 cycles
60°C 4'

cool to 4°C

④ Ethanol precipitation

Each tube add { 2.0 ml 3M NaOAc (pH5.2)
50 ml 95% ethanol

place at RT for 10'

14000 rpm x

Add 250 ml 70% ethanol

14000 rpm x 5'

Dry

18/08/98

Random primer labelling (E68 and A354)

E68 1ml + Hw 105 ml
A354 1ml

↓

95°C 5'

↓

Add 1 ml of dBTP
1 ml of dGTP
1 ml of dTTP
2.5 ml of ^{32}P-dATP
2.5 ml of Primer mix
0.5 ml of Klenow

↓

RT for 1 hr.

↓

~~dH2O~~ 2.5 ml stop buffer

↓

Sephadex column

check concentration

	P^{32} cpm	cpm / µl	5 ml \ need
E68 @	2399285.6	1.2×10^5	41.7 ml
A354 @	3015542	1.5×10^5	3½ 3 ml

21/08/18

Extract the RNA of 8h and 10h stage

two × dilution

	A_{230}	A_{260}	A_{260}/A_{280}	[RNA]	10 μg need	Total volume
8hr.	0.44	0.742	1.6785	3.52 μg/ml	2.84 ml	10 ml (9 ml left)
10hr.	0.579	0.940	1.7063	4.64 μg/ml	2.16 ml	12 ml (11ml left)

22/08/18.

Run RNA gel.

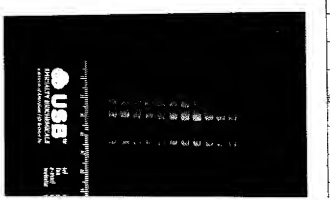
① Making gel

100 ml 1.2% agarose { 1.2 g agarose; 10 ml MOPS; 73 ml H2O; 17 ml 37% formaldehyde }

② RNA samples

	for one sample (15μl)	for 12	
formamide	2.5 ml	90	12.1 ml × 11 tubes
37% formaldehyde	1.3 ml	36	
10× MOPS	1.5 ml	18	
EB	0.4 ml	1.0	
RNA (10μg)	2.9 ml		

	8h	10h	12h	14h	16h	18h	20h	24h	48h	72h	A
10μg	2.84	2.16	2.39	1.95	2.02	2.17	2.34	1.84	1.53	1.28	1.27
H2O	/	0.74	0.51	0.95	/	0.73	0.66	1.06	1.37	1.65	1.63



← 6) membrane

23/08/98.

Labelling (desmin (1 ml of fragment)
| A228 (1 ml of fragment)

	^{32}P-cpm	cpm / ml	5 ml need
Desmin	2204.35.7	1.1×10^5	45.5 ml
A228	200846.6	1.0×10^5	50 ml
Z134	77886.6	0.4×10^5	125 ml

Hybridization (Desmin — ⑤ membrane
| A228 — ⑥ membrane
| Z134 — ① membrane.

25-27.

Redo hybridization desmin on ⑥

E68 on ⑩ — two high background.

E68 on ⑥.

Another two muscle cDNA clones needed to do

α-actin : using 3' UTR

~~MLC 1 (skeletal)~~

MLC 3 (fast skeletal) E94

3/19/98.

1. Amplify E442 (α-actin) 3'UTR

100bp


10 x buffer	5 μl	94°C 5'	
dNTP	2.5	94°C 30"	} 35 cycles
MgCl2	3	55°C 1'	
T7	0.5	72°C 1'	
E442-3	0.5	72°C 5'	
Taq (5u/μl)	0.2		
Template	1 (E442 plasmid 20 x dilution)		
H2O	37.3		
	50 μl		

recover the 350bp into 20 μl H2O

2. Microinjection

MCK-EGFP (500 ng/μl)

injected 23 ~~fish~~ embryos

5 dead

18 alive, no expression

3/19/98.

1. check the recovered band and the MCK (for digestion) plasmid

① ② λ/HindIII (200ng)

① 350bp band. (α-actin 3'UTR) about (3 ng/μl.)

② MCK plasmid (in pBluescript) about (0.25 μg/μl)

2. Digestion

	α-actin 3'UTR	MCK plasmid
DNA	14 μl (45 ng)	10 μl (2.5 μg)
H2O	/	4 μl
10 x buffer (BamHI)	2 μl	2 μl

3. purify fragment digested
for pBluescript, run agarose gel.
Gel purify the 2.9kb fragment into 20ml H_2O
for α-actin 3'UTR, add 150ml of TE buffer,
200ml of phenol, mix, 14000rpm x5'
take the upper 195 ml, add 19.5ml of
3M NaOAc, 400ml pure ethanol
put at -80°C for 2hrs
14000rpm x5' at 4°C, wash with 70% ethanol
redissolve in 15ml of H_2O

4. Ligation
DNA (α-actin 3'UTR B2/KpnI) 15 ml
pBluescript (B2/KpnI) 2ml
10X T4 ligase 2ml
T4 ligase (4u/μl) 0.5ml
RT for 2 hrs, transform DH5α competent cell

5. PCR again to amplify E442 3'UTR

10X PCR buffer	3 ml
2mM dNTP	2.5ml
$MgCl_2$	3 ml
E442.3	0.5ml
T7	0.5 μl
Taq	0.2μl
Template	2 ml
H_2O	23.3ml
	30ml

Gel purify the fragment into 20 μl H_2O

b. Random primer labelling

	Exon 3'UTR	Z94
DNA	4 μl	2 μl
H_2O	6 μl	8 μl

↓

95°C 5'

↓

Add ^{32}P-dATP 4 μl

dCTP 1 μl

dTTP 1 μl

dGTP 1 μl

Primer mix 7.5 μl

Klenow 0.5 μl

↓

RT for 1 hr

Check cpm.

	^{32}P-cpm / 2 μl	cpm / μl	5 μl Hyb buf. need
α-actin	175078	0.875×10^5	17 μl
Z94	207039	1.035×10^5	48 μl

4/9

1. only one colony grew on the plate.
   PCR check using primers E4A2.3 and M13.
   It was the right colony.

2. Inoculate the colony in 5 ml LB/Amp

3. At the same time, do another 3'UTR digestion by BamH1 / Kpn1.
   use 10 ml of PCR (yesterday, fragment (about 100 ng).
   After digestion, incubate at 70°C for 10' to inactivate the enzymes
   use half of the digestion reaction, add 1 ml of digested vector,
   2 ml of 10x ligase buffer, 0.5 ml of ligase (4u/ml), ET for 2 hr.
   Transform DH5α cells.

5/9

1. Plasmid miniprep
   d-actin 3'UTR in pBluescript
   in 25 ml of H2O

2. Single digestion by BamH1.

DNA	10 μl	
10x Buffer	2 ml	
10 x BSA	2 ml	37°C incubation for 2hrs.
BamH1	2 ml	check 0.5 μl on agarose gel.
H2O	4 μl / 20 ml	

3. Ethanol precipitation.
   Add TE to 200 μl. add 200 μl of phenol MIX 14000 rpm x 5'
   take 195 μl of upper liquid. add 19.5 μl of 3M NaOAc, 440 ml of pure ethanol
   -20°C o/N.

6/9.

① 14000rpm × 30', at 4°C

wash with 500 µl 70% etoH, 14000 rpm × 15', at 4°C

air dry.

resolusolve in 10 µl DEPC H_2O

check 0.5 µl on agarose gel.

② Dig-RNA probe synthesis

DNA	4 µl
5× Transcription buffer	4 µl
10× Dig-NTP	2 µl
RNAsin	1 µl
T7 RNA pol	1 µl → 37°C incubation for 2hr.
DTT	1 µl
H_2O (DEPC)	7 µl
	20 µl

10/9.

In situ hybridization

1619

① Make Fluorescent - α-tropomyosin probe

⊗ E71 (BamH1 digested)	5 ml
5X Transcription buffer	4 ml
10X NTP - fluorescat Mix	2 ml
DTT (100mM)	2 ml
RNAsin	1 ml
T7 polymerase	2 ml
DEPC H_2O	4 ml
	20 ml

37°C incubation for 2.5 hrs

↓

Add DNAse 2 ml, 37°C incubation for 15 min

↓

Add 4M LiCl 2.5 ml, 75 ml of pure ethanol -20°C for 1 hr.

↓

14000 rpm x 30', wash with 70% ETOH. 14000 rpm x 15', air dry.

↓

redissolve in 20 ml DEPC water

↓

check 1 ml on formaldehyde gel. (if it is good)

② Select 16-19 h embryos, for whole hybridization

2/19

Check the RNA probe

① E371 fluorescein new 1μl
② E371 fluorescein old 1μl
③ α-Retin - dig 1μl
④ E94 - dig 1μl
⑤ E134 - dig new 1μl
⑥ E134 - dig old 2μl
⑦ E485 - dig new 1μl
⑧ E485 - dig old 2μl

① ② ③ ④ ⑤ ⑥ ⑦ ⑧

In situ

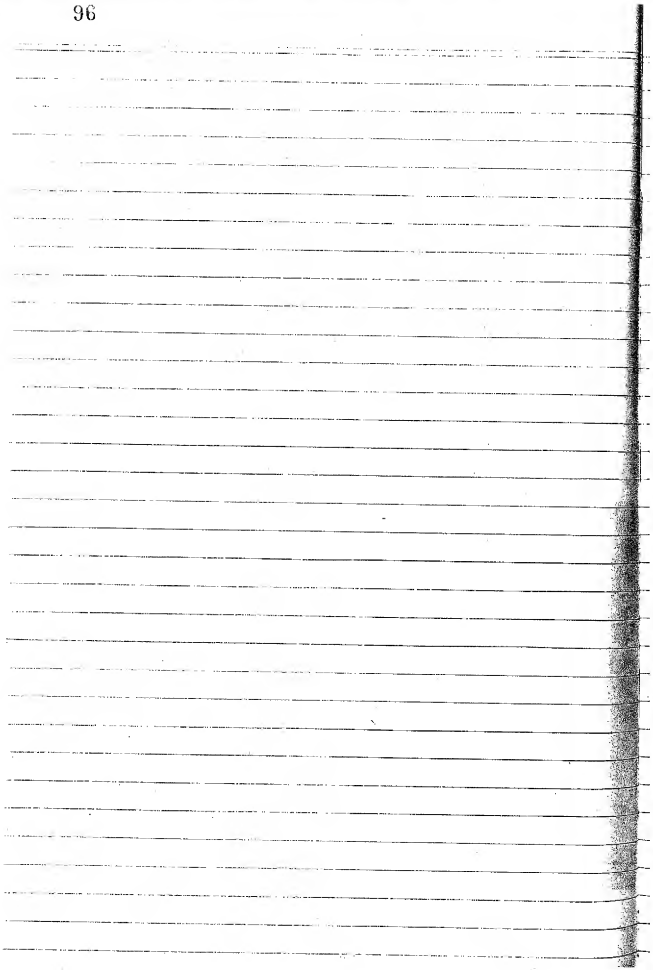
E371-fluo 0.5μl + E134 1μl / or E485 1μl in 200μl Hyb. buf.

In. situ data

	10h.	12h. take again	14h.	16h.	17h	18h	19h.	20h.
E371			✓		(16.5h) ✓	✓	✓	
A324		✓ (flatten needed)	take again ✓	✓				
E496			✓		✓ ✓			
NH62				take again	✓	✓		✓
E68			✓		✓	✓		
E134								✓
E465					✓			
design	take again	✓ take again	✓	✓		take again		
α-actin						✓		
E94								

show number starting

3dh	4dh	
✓	✓	show { fine hair, only MM2 is shown jaw muscle eye muscle
✓	✓	
✓	✓	
✓	✓	show heart. desmin 免疫 (redo)
✓	✓	
✓	✓	
✓ (redo)	✓	
✓ (4-7月A)		
✓ 16	✓	
(5-7月A)		



8/12/98

Northern hybridization on different tissues

1. Labelling
   - E371 (1μl)
   - E68 (1μl)
   - desmin (1μl)
   - MCK (1μl)

Count check

	cpm/μl	cpm/μl	5×10^6 need
E371	3807714.2	1.9×10^5	26.3 μl
E68	604998.6	2.02×10^5	24.8 μl
desmin	411252.8	2.06×10^5	24.3 μl
MCK	491392.4	2.46×10^5	20.3 μl

2. Hybridization

E371	—	7/9/98	A	3.5	4350
E68	—	11/9/98	A	3, 46	E68
desmin	—	11/9/98	B		desmin
MCK	—	7/9/98	B		

E371

11/12/98

labelling { E94 (1μM)
α-actin (4μM)
~~desmin~~ E45 (1N)
~~HMK~~ E134 (1μM)

Count check	cpm/2μl	cpm/1mM	5×10⁶ need
E94	19198.8	9.60×10^{6}	52.1 μl
α-actin	221473.6	1.11×10^{5}	45.0 μl
E45	192023.3	9.60×10^{6}	52.1 μl
E134	171537.0	8.58×10^{6}	58.3 μl

desmin cpm/μl — 5×10⁶ need

$2.06\times10^{5} \times 0.385 = 1.78\times10^{5}$ — 28.1 need

E134 — 7/9 B

E94 — 7/9 A

E45 — 11/9 A

α-actin — 11/9 B

EXHIBIT 10

Asynchronous Activation of 10 Muscle-Specific Protein (MSP) Genes During Zebrafish Somitogenesis

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ABSTRACT **In the present study, 10 zebrafish cDNA clones coding for muscle-specific proteins (MSPs) were characterized and most of them encode fast skeletal muscle isoforms. They are skeletal muscle α-actin (*acta1*), fast skeletal muscle a-tropomyosin (*tpma*), fast skeletal muscle troponin C (*tnnc*), fast skeletal muscle troponin T (*tnnt*), fast skeletal muscle myosin heavy chain (*myhz1*), fast skeletal muscle myosin light chain 2 (*mylz2*), fast skeletal muscle myosin light chain 3 (*mylz3*), muscle creatine kinase (*ckm*), parvalbumin (*pvalb*), and desmin (*desm*). Using these cDNA probes, their expression patterns in developing embryos and adults were compared by Northern blot hybridization and whole-mount in situ hybridization. All of the 10 genes are expressed in both embryos and adult fish, and the expression is highly abundant in skeletal muscle. Among them, *acta1, tpma, tnnc, tnnt, myhz1, mylz2, mylz3* and *pvalb*, are expressed specifically in fast skeletal muscle while *ckm* and *desm* are expressed in both fast and slow skeletal muscles. In addition, *tpma, ckm,* and *desm* are also expressed in the heart. Ontogenetically, the onset of expression of these MSP genes in zebrafish skeletal muscle varies and the expression occurs rostral-caudally in developing somites. Shortly after the expression of *myoD, desm* is the first to be activated at ~9 hpf, followed by *tpma* (~10 hpf), *tnnc* (~12 hpf), *acta1* (~12 hpf), *ckm* (~14 hpf), *myhz1* (~14 hpf), *mylz2* (~16 hpf), *mylz3* (~16.5 hpf), *tnnt* (~16.5 hpf), and *pvalb* (~16.5 hpf). At later stages (after 48 hpf), these MSP genes are also expressed in fin buds and head muscles including eye, jaw, and gill muscles. Thus, our experiment demonstrated the order of expression of the 10 MSP genes, which may reflect the sequence of muscle filament assembly. In spite of the asynchrony in activation of these MSP genes, the timing of expression for each individual MSP gene appears to be synchronous to somite development as each somite has an identical timetable to express the set of MSP genes.** © 2000 Wiley-Liss, Inc.

Key words: actin; tropomyosin; troponin; myosin heavy chain; myosin light chain; creatine kinase; parvalbumin; desmin; MyoD, skeletal muscle; cranial muscle; cardiac muscle

INTRODUCTION

Muscle is a popular model system for investigation of the mechanism of tissue-specific gene expression. Many proteins are expressed uniquely in muscle and are called muscle-specific protein (MSP) in this study. These include contractile proteins (e.g., α-actins, myosins, tropomyosins, troponins, and so on) as well as soluble muscle proteins and enzymes (e.g., parvalbumin and creatine kinase). A common feature of many of these proteins is that they have many isoforms, which are generated either from separate genes or by different splicing of the same gene. These isoforms are expressed in different muscle fiber types or even in the same muscle fiber at different development stages. For example, there are at least 10 skeletal myosin heavy chain isoforms in mammals (McKoy et al., 1998). Different myosin heavy chains are expressed in embryonic, neonatal, and adult muscle fibers, as well as in fast or slow muscle fiber types (Ontell et al., 1995; McKoy et al., 1998).

In vertebrates, muscles make up much of the mass of the body, and are present in close association with many organs. On the basis of their structure and function, muscles can be classified into three types: skeletal muscle, smooth muscle, and cardiac muscle. During vertebrate embryogenesis, skeletal, cardiac, and smooth muscle cells arise from different mesodermal precursors in different regions of the embryo. The skeletal muscle is derived from somites, except for some head muscles that appear to arise from cephalic mesoderm. The molecular mechanism of muscle cell differentiation has been well characterized (for review, see Molkentin and Olson, 1996; Olson and Klein, 1994). Two classes of transcription factors, the myogenic basic helix-loop-helix proteins including MyoD, Myogenin, Myf5, and MRF4, and several members of MEF2 (myocyte-specific enhancer factor 2) family, play a crucial role in the process. It has been proposed that *myoD* and *myf5* are muscle determination genes and are expressed in proliferating myoblasts while *myogenin* and *MRF4* are differentiation genes, which are not expressed until myoblast exit the cell cycle in response to

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Received 4 November 1999; Accepted 15 June 2000
Published online 1 September 2000

mitogen depletion (Olson and Klein, 1994). MEF2 is also a key regulator for both the skeletal and cardiac muscle lineages and may directly control myogenic bHLH genes (Molkentin and Olson, 1996). When muscle cells are finally differentiated, the set of MSP genes are expressed. Both MEF2 and myogenic bHLH transcription factors are directly involved in activation of these MSP genes (Kaushal et al., 1994; Molkentin et al., 1995).

As muscle contractile filaments consist of dozens of distinct MSPs, how the genes encoding these proteins are coordinately regulated to produce the pool of MSPs for myofibril assembly remains unclear. Although detailed expression patterns have been described for some individual MSP genes (Ontell et al., 1995) and expression of selected MSPs were compared in in vitro cultured muscle cells (Gunning et al., 1987; Lin et al., 1994), no systematic comparison of MSP gene expression has been conducted in any *in vivo* developing system.

The zebrafish, *Danio rerio*, is particularly feasible for analysis of muscle-specific gene expression. The embryonic expression in skeletal muscle is easily observable, and an adequate amount of muscle tissue is available from adult fish for molecular and biochemical analyses. More importantly, it has been demonstrated that the fish skeletal muscle development generally follows patterns typical of all vertebrates (Fishman et al., 1996). Previously, by an EST approach, we have identified and isolated over 700 distinct zebrafish cDNA clones from nearly 3,000 partially sequenced clones (Gong et al., 1997; Gong, 1999; and unpublished data). Many of these identified clones encode zebrafish MSPs. In this study, 10 zebrafish MSP cDNA clones, most of which encode skeletal muscle isoforms, were selected and used for comparative studies of their expression patterns in skeletal muscle development in zebrafish. We found that these MSP genes are activated asynchronously and follow a temporal order. Thus, these genes may be used as molecular markers for different stages of skeletal muscle development.

RESULTS

Muscle Specific Protein (MSP) cDNA Clones

Ten MSP cDNA clones were chosen for this study. Among the 10 clones, 9 were our EST cDNA clones derived from either an embryonic (E) or an adult (A) cDNA library (Gong et al., 1997). They are E442 (*acta1* codes for a skeletal muscle α-actin), E371 (*tpma* for a fast skeletal muscle α-tropomyosin), A354 (*tnnc* for a fast skeletal muscle troponin C), E134 (*tnnt* for a fast skeletal muscle troponin T), E68 (*myhz1* for a fast skeletal muscle myosin heavy chain), E94 (*mylz3* for a fast skeletal muscle *myosin light chain 3*), E72 (*mylz2* for a fast skeletal muscle myosin light chain 2), E146 (*ckm* for a muscle creatine kinase), and E465 (*pvalb* for a parvalbumin). A *desmin* cDNA clone (*desm*), which was isolated by screening the same zebrafish embryonic library, was also included. Seven of the cDNA clones (*acta1, tpma, tnnc, tnnt, myhz1, mylz2* and *mylz3*) encode myofibril contractile proteins, which include most of the major contractile proteins in the thin and thick filaments. The *desm* clone encodes a type III intermediate filament protein that links myofibrils into bundles in muscle cells (Yang and Makita et al., 1996). *ckm* and *pvalb* encode soluble proteins. Muscle creatine kinase is a small soluble enzyme and is crucial in generating ATP from stores of creatine phosphate during muscle contraction (Trask et al., 1988), while parvalbumin, an acidic Ca^{++}- and Mg^{++}-binding protein, acts as a cytosolic Ca^{++} buffer and plays a role in muscle relaxation (Pette and Staron, 1990). All selected cDNA clones were sequenced completely. Except for the *myhz1* clone, all contain full-length coding region. The sequence information and their Genbank access numbers are summarized in Table 1. The full-length sequence of *mylz2* (previously named *MLC2f*) and *desm* cDNAs has been reported previously (Xu et al., 1999; Loh et al., 2000). The *tpma* cDNA sequence is essentially identical to the previously reported *tropomyosin* cDNA sequence (Ohara et al., 1989) and thus may be derived from the same zebrafish gene. The rest of seven zebrafish cDNA sequences are reported for the first time.

Tissue Distribution of MSP mRNAs

To examine the tissue distribution of mRNAs from the 10 selected MSP genes, Northern blot hybridization was carried out. Total RNAs were prepared from several adult tissues including the heart, brain, eyes, gills, intestine, liver, trunk skeletal muscle, ovary, skin, and testis. As shown in Figure 1, all of the 10 genes were expressed predominantly or specifically in the trunk skeletal muscle. For *tnnc, acta1, myhz1, mylz2, mylz3, tnnt*, and *pvalb* genes, their mRNAs were only detected in the trunk skeletal muscle; while *desm, tpma*, and *ckm* mRNAs were also presented in the heart. In some cases, faint hybridization signals were also detected in the skin, which was likely due to contamination with muscle tissue when the skin sample was prepared. To monitor the even-loading of RNA samples from different tissues, an identical blot was made and hybridized with a ubiquitous probe derived from a β-*actin* cDNA clone (our EST clone E43). As shown at the bottom of Figure 1, a 1.8-kb transcript was detected in all tissues. In the heart and the skeletal muscle samples, a second transcript of about 1.5 kb was also detected and they were likely derived from the cardiac and skeletal α-*actin* genes, respectively, due to cross-hybridization with the β-*actin* cDNA probe.

Developmental Accumulation of MSP mRNAs

To examine the temporal expression of the 10 MSP genes, total RNAs were extracted from 8 different stages of zebrafish embryos from 12 hpf (6-somite stage) to 72 hpf (hatched fry), as well as from adult fish. Northern blot hybridization was then performed. As shown in Figure 2, expression of *desm* was the first to

TABLE 1. Summary of the MSP cDNA Clones[a]

Clone no.	Gene names	Gene abbreviations	Most homologous cDNA (species, Genbank access no.)	AA sequence identity (%)	Insert length (nt)	Coding region (aa) (F, full length)	Genebank accession no.
E442	*actin, alpha 1, skeletal muscle*	*acta1*	Skeletal α-actin (carp, D50028)	99.7	1,284	377 (F)	AF180887
E371	*alpha tropomyosin*	*tpma*	Fast skeletal muscle α-tropomyosin (zebrafish, M24635)	100	1,246	284 (F)	AF180892
A354	*troponin C, fast skeletal muscle*	*tnnc*	Fast skeletal troponin C (Xenopus, AB003079)	82.4	970	160 (F)	AF180890
E134	*troponin T, fast skeletal muscle*	*tnnt*	Fast myotomal muscle troponin T (salmon, AF072687)	80.3	1,098	230 (F)	AF180889
E68	*myosin, heavy polypeptide 1, fast skeletal muscle*	*myhz1*	Fast skeletal muscle myosin heavy chain (carp, D89992)	91.9	1,346	423	AF180893
E94	*myosin, light polypeptide 3, fast skeletal muscle*	*mylz3*	Fast skeletal muscle myosin light chain 3 (carp, AI353819)	94.0	946	151 (F)	AF180891
E72	*myosin, light polypeptide 2, fast skeletal muscle*	*mylz2*	Fast skeletal muscle myosin light chain 2 (rabbit, M21983)	85.7	1,386	169 (F)	AF081462
E146	*creatine kinase, muscle*	*ckm*	Muscle creatine kinase (carp, AF055288)	94.5	1,542	381 (F)	AF134852
E465	*parvalbumin*	*pvalb*	Parvalbumin (salmon, X97825)	79.8	606	109 (F)	AF180888
Desmin	*desmin*	*desm*	Desmin (chicken, AB011672)	60.1	1,798	473 (F)	U47113

[a] All gene names and abbreviations have been approved by the Zebrafish Nomenclature Committee.

be detected among the 10 MSP genes. The *desm* mRNA appeared prior to 12 hpf and increased slightly during development. The expression of other 9 MSP genes was initiated later and increased rapidly during the early embryonic development. The *tpma*, *tnnc*, and *acta1* mRNAs started to appear in embryos at ~14 hpf, followed by the *ckm* mRNA at ~18 hpf, the *mylz2* mRNA at ~20 hpf; Finally, the *myhz1*, *mylz3*, *tnnt*, and *pvalb* mRNAs were expressed between 20 to 24 hpf. Another ubiquitous probe derived from an *acidic ribosomal phosphoprotein P0* (*arp*) cDNA clone, which was expressed constantly during this period of embryogenesis (Ju et al., 1999), was hybridized to an identical RNA blot to monitor the quantity of RNA and to ensure the even loading of all RNA samples (Fig. 2, bottom). It is noteworthy from Figure 2 that almost all of these MSP genes were expressed weaker in adult fish than in developing embryos, except for the *ckm* gene, whose expression increased steadily from embryo to adult.

Therefore, as demonstrated in Figures 1 and 2, these MSP genes appear to be expressed in both developing embryos and adults. This conclusion is also supported by the fact that at least eight of the 10 MSP genes, *tpma*, *tnnc*, *acta1*, *ckm*, *myhz1*, *mylz2*, *tnnt*, and *pvalb*, were represented by EST clones from both embryonic and adult cDNA libraries (Gong et al., 1997; unpublished data). It is well known that many of the MSP genes have isoforms differentially expressed temporally and spatially (e.g., Ennion et al., 1999; Lu et al., 1999). Although there is no indication of cross hybridization using our probes under our hybridization conditions, we can not completely rule out the possibility of the presence of highly homologous isoforms that might hybridize to our probes. This will be clarified when more MSP cDNA clones are available in the future.

Ontogenetic Expression of MSP Genes During Somitogenesis

To investigate the detailed expression patterns of the 10 MSP genes in developing embryos, whole-mount in situ hybridization was carried out with embryos of various somitogenesis stages. Because the expression of *myoD* mRNA has been well characterized (Weinberg et al., 1996), the *myoD* probe was also included for the comparative study. As reported by Weinberg et al. (1996), *myoD* mRNA was first detected around 7 hpf in adaxial cells as two continued lines along the notochord before somite formation, as illustrated by an example of an 11 hpf embryo hybridized with the *myoD* probe (Fig. 3A–C). Similarly, *desm*, the earliest MSP gene we examined, were also expressed in adaxial cells about 2 hr after *myoD* expression at about 9 hpf. An example of early expression of *desm* at 11 hpf is shown in Figure 3D–F. By 13 hpf (8-somite), *myoD* expression was extended laterally in formed somites as well as in presomitic regions (Fig. 3G), while at the same stage the

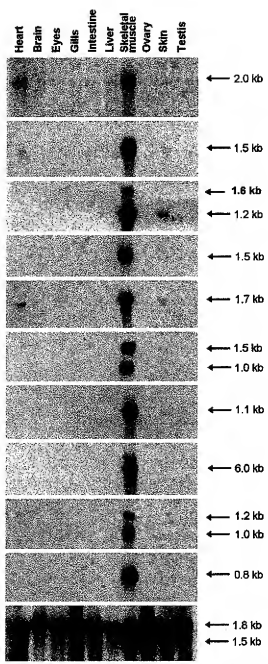

Fig. 1. Tissue distribution of the 10 MSP mRNAs in adult zebrafish. Total RNAs were prepared from various adult tissues as indicated at the top of each lane and fractionated by formaldehyde-agarose gel electrophoresis. Ten micrograms of RNA were loaded in each lane and hybridized with individual MSP cDNA probes as indicated on the left of each panel. The sizes of major hybridized transcripts are indicated on the right. An identical RNA blot was hybridized with a ubiquitously expressed β-*actin* probe to monitor the quantity and quality of the RNAs (bottom).

lateral extension of *desm* expression was much less and limited only to the formed somites (Fig. 3I). Cross-sectioning of the in situ hybridized embryos indicated that the expression of *myoD* was extended also more dorsally than *desm* expression (Fig. 3H,K). The second earliest MSP gene, *tpma*, showed a similar expression pattern as that of *desm* (Fig. 3M–O).

For a thorough comparison of the expression patterns of the 10 MSP genes, the lateral view of the in situ hybridized embryos at various somitogenesis stages is shown in Figure 4 and the information about their expression is summarized in Tables 2 and 3. All of the 10 MSP genes were activated rostral-caudally in developing somites. Consistent with the observation from the northern blot hybridization (Fig. 2), *desm* mRNA was the first to be detected and appeared at ~9 hpf. From 10 to 12 hpf, when the first anterior 6 somites were rapidly formed, the *desm* transcript signal was intensified and extended more posteriorly (Fig. 4A,B). At 10 hpf, *tpma* mRNA started to be detected and the signal became more intensified at 12 hpf (Fig. 4F). Another MSP gene, *tnnc*, started to express at the same position as two faint lines at 12 hpf (Fig. 4J).

Starting from 12 hpf until 30 hpf, two somites are formed each hour in a regular interval (Hanneman and Westerfield, 1989; Kimmel et al., 1995; Westerfield, 1994). The number of lateral bands of cells containing *desm*, *tpma*, and *tnnc* mRNAs increased with the increase of somites number. By 14 hpf, when 10 somites were formed, the *desm* and *tpma* transcripts were detected in all of the 10 somites (Fig. 4C,G), while strong expression of *tnnc* transcript was observed in the first 8 somites and faint expression in the last 2 somites (Fig. 4K). Similarly, *acta1* transcript was also observed as 8 strong bands and 2 faint bands (Fig. 4N), suggesting that *acta1* was activated at about the same time as *tnnc*, though its transcript was not detected until 14 hpf stage, probably because the initial expression in the first few somites was too weak to be detected using a short 3′ UTR probe (180 bp). Both *tnnc* and *acta1* were also expressed in adaxial cells prior to somite formation.

At 14 hpf (10-somite), *ckm* and *myhz1* mRNAs were detected only in the first 6–7 somites (Fig. 4Q,T). Like the early four MSP genes, *myhz1* was also expressed in the posterior adaxial cells in the unsegmented region (Fig. 3P). However, the *ckm* mRNA was detected only after the formation of somites, and there was no apparent expression in the unsegmented lateral mesoderm (Fig. 3Q). Other MSP genes, including *mylz2*, *mylz3*, *tnnt*, and *pvalb*, showed a similar expression pattern as *ckm* but were activated later. *mylz2* mRNA was first detected at 16 hpf (Fig. 4W) while the transcripts of the other three MSP genes, *mylz3*, *tnnt*, and *pvalb*, were not detected until 16.5 hpf (15-somites) (Fig. 4Y and data not shown).

Therefore, it is apparent that these MSP genes are asynchronously activated during somitogenesis. In general, the in situ hybridization data are consistent with the observation made by Northern hybridization in terms of the temporal expression programs of these MSP genes, except the followings. First, for all the genes studied, the initial detection of their expression

is earlier by in situ hybridization than by Northern blot hybridization, apparently because the in situ hybridization approach is more sensitive. Second, while in Northern blot hybridization, the *myhz1* transcript is detected later than *mylz2* and *mylz3*, in situ hybridization reveals that it is expressed slightly earlier than *mylz2* and *mylz3*. The simple explanation might be that the initial expression of *myhz1* mRNA is too low to be detected by Northern blot hybridization.

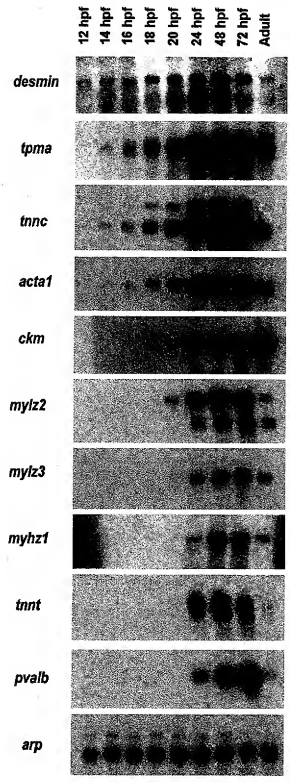

Fig. 2. Developmental accumulation of the 10 MSP mRNAs in developing zebrafish embryos. Total RNAs were prepared from zebrafish embryos of various stages from the beginning of somitogenesis (12 hpf) to hatched fry (72 hpf) and fractionated by formaldehyde-agarose gel electrophoresis. Ten micrograms of RNA were loaded in each lane and hybridized with individual MSP cDNA probes as indicated at the left of each panel. The stages of embryos are indicated at the top of each lane; adult, RNA was prepared from whole adult fish. An identical RNA blot was hybridized with a ubiquitously expressed *acidic ribosomal phosphoprotein* (*arp*) probe to monitor the quantity and quality of the RNAs (bottom).

Based on whole-mount in situ hybridization (Fig. 4 and data not shown), the numbers of somites showing positive hybridization signals for each of the 10 MSP probes are summarized for various stages of somitogenesis (Table 2). Several interesting conclusions can be drawn from this comparison. First, the number of somites expressing a given MSP gene increased at about the same rate as the number of somite formed during somitogenesis. For example, from 16.5 hpf to 18.5 hfp, 4 new somites are formed and the number of somites expressing each MSP mRNA also increases by ~4. Second, for each MSP probe, the number of posterior somites negative for the hybridization signal remains constant during somitogenesis. For example, there were two and four unhybridized somites for the *ckm* and *mylz2* probes, respectively, and these numbers remain the same from 14-somite to 19-somite stages. However, when a MSP gene is just activated in the first 6–7 somites, the signal is generally weak and thus the number of hybridized somites may be underestimated. Therefore, the activation of these MSP genes appears to be synchronous to the differentiation state of developing somites. Hence, the number of somites expressing certain MSP gene can be used as an index to determine the order and timing of MSP gene expression. Because the pace of formation of a new somite is constant at one somite per half hour between 12–25 hpf, we, based on the number of unhybridized somites and the initial timing of activation, can deduce the timing of the MSP gene activation relative to the formation of a somite (Table 3). The time span for activation of these 10 MSP genes is 10 hr, duration of formation of 20 somites. The earliest MSP gene, *desm*, is activated about 4 hr prior to somite formation whereas the latest, *pvalb*, 6 hr after somite formation.

Expression of MSP Genes in Fast and Slow Muscles

Different MSP genes also show different expression regions in zebrafish skeletal muscle. *acta1*, *tpma*, *tnnc*, *myhz1*, *mylz2*, *mylz3*, *tnnt*, and *pvalb* transcripts were detected only in the fast skeletal muscle, as exemplified

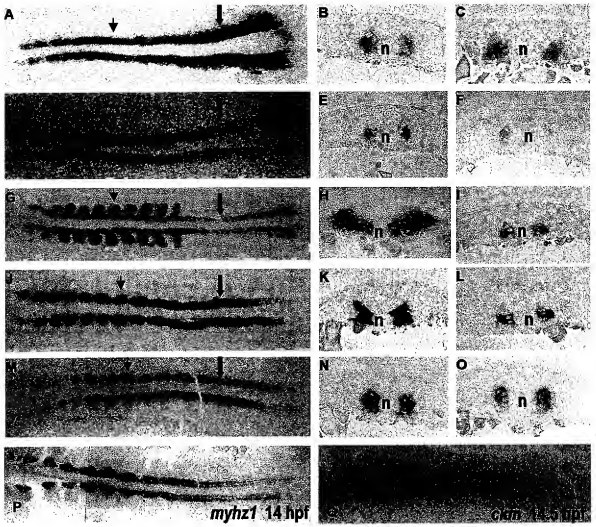

Fig. 3. Comparison of expression of *myoD* (**A–C, G–I**) and selected MSP genes, desmin (**D–F, J–L**), *tpma* (**M–O**), *myhz1* (**P**), and *ckm* (**Q**). Whole-mount in situ hybridization using antisense riboprobes was carried out as described in Experimental Procedures. Following in situ hybridization, selected embryos were cross-sectioned using a cryostat. The probes and stages of embryos are indicated on the right or inside the pictures. A, D, G, J, M, P, and Q are dorsal view of flatted specimens. B, E, H, K, and N are sections of embryos in the mid-part as indicated by short arrows in the flatted specimens, while C, F, I, L, and O are sections of the posterior part of the embryos as indicated by long arrows. n, notochord or notochord precursor.

in Figure 5 A–D and M–P for *mylz2* and *myhz1*, respectively. No expression was detected in already migrated slow skeletal muscle cells, which were determined by the lack of staining in the horizontal myoseptum that contains muscle pioneers, a subset of slow muscle cells (side view in Fig. 5B,N), and by the lack of staining in the monolayer of superficial muscle cells (Fig. 5C,O), as defined by Devoto et. al. (1996) using an antibody against a slow muscle myosin. One of our EST clones (A14, *smbpc*) encoding a slow myosin binding protein C was also used for in situ hybridization to define slow muscle and the result is shown in Figure 5I–L. In contrast, *desm* mRNAs were expressed in both the superficial muscle cells and the deep portion of the myotome (Fig. 5E,F). Similar results were also observed for *ckm* mRNA (data not shown). Thus, *desm* and *ckm* were expressed in both fast and slow muscles. To confirm the expression in fast and slow muscles, two-color in situ hybridization was carried out using a MSP probe and the *smbpc* probe (Fig. 5D,H,L,P). In addition, *myhz1* was also expressed in the transverse myosepta but not in the horizontal myosepta (Fig. 5N).

Expression of MSP Genes in Late Embryogenesis

Developmental expression of the 10 zebrafish MSP genes was also examined in late embryogenesis up to 72 hpf. All the 10 mRNAs were detected in fin buds and head muscles including eye, jaw, and gill muscles, which, like the trunk deep skeletal muscle, are also

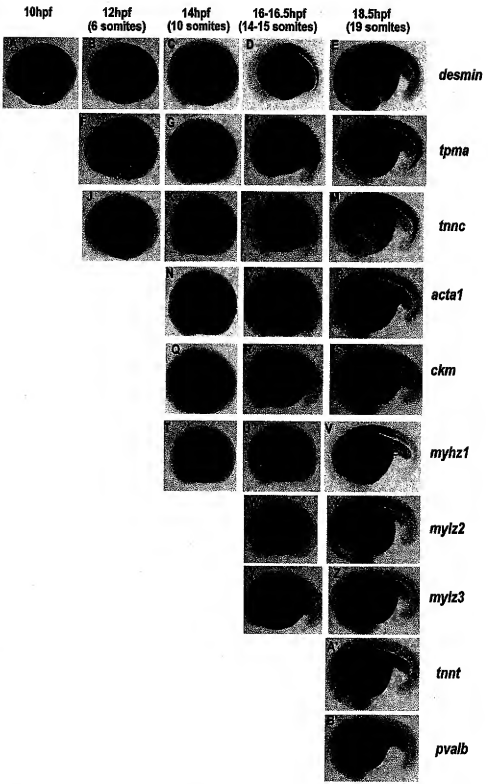

Fig. 4. Ontogenic expression of the 10 zebrafish MSP genes as detected by whole-mount in situ hybridization. Embryos were hybridized with *desm* (**A–E**), *tpma* (**F–I**), *tnnc* (**J–M**), *acta1* (**N–P**), *ckm* (**Q–S**), *myhz1* (**T–V**), *mylz2* (**W, X**), *mylz3* (**Y, Z**), *tnnt* (**A'**), and *pvalb* (**B'**) probes, respectively. Vertical panel columns show embryos at the same stage: 10 hpf (A), 12 hpf (B, F, J), 14 hpf (C, G, K, N, Q, T), 16 hpf (O, U, W), 16.5 hpf (D, H, L, R, Y), and 18.5 hpf (E, I, M, P, S, V, X, Z, A', B'). Embryos are viewed laterally, anterior to the left, and are at the same magnification.

TABLE 2. Numbers of Somites Expressing MSP mRNAs as Detected by Whole Mount In Situ Hybridization[a]

MSP genes	10 hpf	12 hpf (6-somites)	14 hpf (10-somite)	16.5 hpf (15-somite)	18.5 hpf (19-somite)	20 hpf (22-somite)
desm	+	6+	10+	15+	19+	22+
tpma	+	6+	10+	15+	19+	22+
tnnc		Faint	10+	15+	19+	22+
acta1			10+	15+	19+	22+
ckm			7	13	17	20
myhz1			7	13	17	ND
mylz2				8	13	16
mylz3				7	13	ND
tnnt				Faint	7	12
pvalb				Faint	7	10

[a] +, expression was also found in the posterior unsegmented region; ND, not determined.

TABLE 3. Timing and Specificity of MSP Gene Activation in Zebrafish Embryos[a]

MSP genes	Initial detection (hpf)	No of somites unstained	Timing of activation (hr)	Types of expressing muscles[a]
desm	~9	0	(~4)	F, S, C
tpma	~10	0	(~3)	F
tnnc	~12	0	(~1)	F
acta1	12–14	0	(~1)	F
ckm	~14	2	~1	F, S, C
myhz1	~14	2	~1	F
mylz2	~16	6	~3	F
mylz3	~16	6	~3	F
tnnt	~16.5	10	~5	F
pvalb	~16.5	12	~6	F

[a] Timing of activation relative to the formation of the somite (0 h). Hours in parentheses indicate the time before the formation of visible somites. [a]F, fast skeletal muscle; S, slow skeletal muscle; C, cardiac muscle.

striated fast muscles. Their expression in these areas is exemplified in Figure 6A and B, where a 72-hpf embryo was hybridized with the *mylz2* probe; this pattern on head muscles is essentially identical to that described by Schilling and Kimmel (1997). The other nine MSP mRNAs also showed the same pattern of expression at this stage (date not shown). As the 10 MSP genes are expressed sequentially in somite muscles, the same order of sequential expression is probably also true for head muscles. For example, at 60 hpf, *desm* mRNA (the earliest) was presented in several pairs of head muscles including adductor mandibulae (am), medial rectus (mr), intermanibularis anterior (ima), intermandibularis posterior (imp), and interhyideus (ih) (Fig. 6C); while *pvalb* mRNA (the latest) was detected only in am (Fig. 6D). Ontogenetically, am appears at 53 hpf, mr and ih at 58 hpf, and ima and imp at 62 hpf (Schilling and Kimmel, 1997); thus, it is apparent that *desm* is expressed earlier than *pvalb* in these head muscles. The differential expression of other intermediate MSP genes was difficult to capture as the time interval of appearance of a pair of new head muscles was generally more than 4 hr, which was much longer than the time difference of the expression of different MSP genes as measured in the rapidly developing somite muscles (one somite per 0.5 hr).

In addition, *desm* and *ckm* transcripts were also detected in the heart (Fig. 6E and F), which is consistent with the Northern blot data on tissue distribution of MSP mRNAs in adult fish. However, although *tpma* mRNA was detected in adult heart tissues (Fig. 2), no expression was detected in the embryonic heart up to 72 hpf.

DISCUSSION

Asynchronous Expression of MSP Genes

In this study, 10 zebrafish cDNA clones encoding 10 different MSPs were selected and characterized. All of the 10 MSP genes are specifically or predominantly expressed in skeletal muscle of both embryos and adult fish. However, the initial activation time of these MSP genes in zebrafish skeletal muscle development varies from 9 to 16.5 hpf. As demonstrated by whole-mount in situ hybridization, *desm* was the first to be activated at ~9 hpf, followed by *tpma*, *tnnc*, *acta1*, *ckm*, *myhz1*, *mylz2*, *mylz3*, *tnnt*, and *pvalb*, in this order. Based on the timing and pattern of their expression during somitogenesis, the 10 MSP genes can be roughly classified into three groups. *desm*, *tpma*, *tnnc*, and *acta1* belong to the early gene group and they are expressed in all formed somites and also in adaxial cells prior to and shortly after somite formation. *ckm*, *myhz1*, *mylz2* and *mylz3* are intermediate genes and their expression is absent in the last 2–6 formed somites; i.e., they are activated within 3 hr after somite formation. *tnnt* and *pvalb* are late genes and their expression occurs 5–6 hr after somite formation (Table 3). Thus, these MSP cDNA clones can be used as molecular markers for different stages of skeletal muscle development.

Despite the ontogenetic asynchrony in activation of the 10 MSP genes, the program of activation of these genes seems to be synchronous to the differentiation of somite and thus each somite likely follows an identical gene activation program for the 10 sequentially expressed MSP genes. The same order of expression of the 10 MSP genes is probably maintained for other skeletal muscles such as head muscles. From the ex-

pression sequence of the MSP genes, it is interesting to note that almost all MSP genes encoding thin filament proteins (actin, tropomyosins, and troponin C) are expressed earlier than the genes for thick filament proteins (myosin heavy chain and light chains) and muscle creatine kinase, an enzyme mainly associated with the thick filaments (Otsu et al., 1989). Whether this order of expression also reflects the assembly sequence of skeletal muscle filament will be of interest to determine. It has been suggested that thin and thick filaments assemble independently in muscle cells and it is likely that the thin filaments appear earlier than the thick filaments (Epstein and Fischman, 1991; Holtzer et al., 1997). Thus, our data are consistent with the model of myofibril assembly. Unexpectedly, the mRNA for another thin filament protein, troponin T, appeared quite late, which may indicate that it is not required for early assembly of the thin filaments. Alternatively, it is also possible that there is another copy of zebrafish gene for troponin T that may be expressed earlier. Because the first muscular contractions occur at 17-somite stage (17.5 hpf) (Kimmel et al., 1995), which is shortly after the ontogenetic activation of all the 10 MSP genes characterized, it is possible that zebrafish skeletal muscle is not functional until all the structural proteins are synthesized.

It is also interesting to note that several early MSP genes, including *desm*, *tpma*, *tnnc*, *acta1* and *myhz*, are expressed in adaxial cells even before the formation of somites and their migration. These adaxial cells would migrate to the superficial layer of somite and form slow muscle shortly after somite formation (Devoto et al., 1996). In the well-formed somites, however, only *desm* mRNA was detected in the superficial slow muscle layer, where no transcript from *tpma*, *tnnc*, *acta1*, and *myhz1* was detected. This observation indicated that the differentiating slow muscle initially expressed fast muscle isoforms of these proteins and ceased the expression during or after cell migration. Because the intermediate and late MSP genes are expressed only after the start of migration, it is not clear whether these fast isoform genes are also initially expressed in differentiating slow muscle.

Among these genes studied, *desm* is of much interest because the initiation of its expression in zebrafish embryos occurs much earlier than other MSP genes, even before the formation of somites. In fact, previous studies have revealed that *desmin* is one of the earliest known myogenic markers and one of the first muscle-specific proteins to appear during mammalian embryonic development (Buckingham, 1992). Comparison of amino acids sequence of Desmins from different species indicates that they contain a potential bHLH domain, which shares significant homology with the bHLH domains of the myogenic transcription factors of MyoD type, implying that Desmin could be directly or indirectly involved in muscle gene regulation (Li and Capetanaki, 1994). This possibility was supported by the antisense RNA inhibition experiment. When *desm* antisense mRNA was injected into mouse embryos, myogenic differentiation, myoblast fusion, and myotube formation were all inhibited, coupled with the down-regulation of myogenic factors, MyoD and Myogenin (Li et al., 1994). The fact that zebrafish Desmin shares the same homology in the myogenic bHLH domain (data not shown) and that initiation of its transcription precedes appearance of other MSP mRNAs during development suggests that Desmin may play the same regulatory role in the piscine system.

pvalb is another gene of interest. Two subclasses of parvalbumins have been described, α (pI above 5.0) and β (pI below 4.5) (Goodman and Pechere, 1977). Both α and β parvalbumins are found in muscle tissues of fish and frog, the lower and cold-blooded vertebrates. In contrast, chicken, rabbit, rat, and human muscle tissues express only the α form. Whereas α-parvalbumin is abundant in fast-twitch muscle fibers of the rat and mouse, human muscles only contain very low concentrations of α-parvalbumin, and strong α-parvalbumin expression was found in non-muscle tissues such as brain and kidney (Föhr et al., 1993). Chicken β-parvalbumin (avian thymic hormone; Brewer et al., 1991; Kuster et al., 1991) is expressed in thymus and blood but not in muscle. In this study, a zebrafish *parvalbumin* was identified and according to its sequence alignment with carp and *Xenopus*, it is likely a β isoform. The zebrafish *pvalb* is expressed predominantly in fast skeletal muscle and its transcription is initiated at a late stage of myogenesis. The same phenomenon was observed for chicken muscle *parvalbumin*, which is not detectable in the leg muscles until just before hatching, lagging behind of the synthesis of most contractile proteins (Lepeuch et al., 1979). It has been proposed that the delayed expression of chicken parvalbumin may prevent muscle contraction while the embryo is in the eggshell (Kay et al., 1987). Consistent with this, zebrafish *pvalb* is activated later than most, if not all, MSP genes and its activation (16.5 hpf) correlates well to the first contraction of skeletal muscle in zebrafish embryos (17.5 hpf) by consideration of the lag time for protein translation from *pvalb* mRNA.

Comparison of Expressions of MSP Genes and Myogenic Transcription Factor Genes

There are two major classes of myogenic transcription factor that are critical for activation of MSP genes. One is myogenic basic helix-loop-helix (bHLH) proteins such as MyoD and Myogenin. The other is MEF2 (myocyte-specific enhancer factor 2) family of MADS-box transcription factors (Molkentin et al., 1995; Molkentin and Olson, 1996). Several members from both classes have been studied previously in zebrafish (Weinberg et al., 1996; Ticho et al., 1996). The expression of *myoD* is initiated in the embryos at 7–7.5 hpf, and of *myogenin* at 10.5 hpf (Weinberg et al., 1996). Three *MEF2* genes,

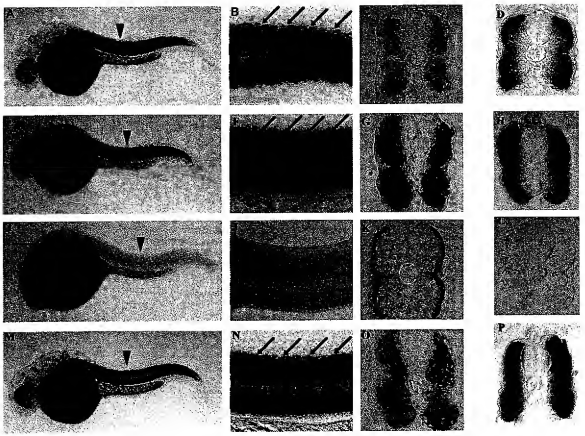

Fig. 5. Expression of *mylz2* (**A–D**, 36 hpf), *desm* (**E–H**, 36 hpf), smbpc (**I–L**, 24 hpf) and *myhz1* (**M–P**, 36 hpf) mRNAs. B, F, J, and N are the enlargement of the mid-part of the embryos shown in A, E, I, and M, respectively. C, G, K, and O show the cross section at the position as indicated by arrowheads in A, E, I, and M, respectively. D, H, and P are two-color in situ hybridization pictures where slow muscles are shown in red (probed with fluorescein labeled *smbpc* antisense RNA) and fast muscles are shown in purple. L is a section of an embryo hybridized with the red fluorescein labeled *smbpc* probe only. The position of transverse myoseptа between somites are indicated by arrows in B, F, and N.

MEF2A, *MEF2C*, and *MEF2D*, have been characterized in zebrafish. *MEF2D* mRNA is the first among the three to be detected, appearing at 8.5 hpf; while *MEF2A* mRNA is first detected at 10 hpf and *MEF2C* mRNA at 12 hpf (Ticho et al., 1996). The expression of these myogenic transcription factors shares the same spatial pattern with those early MSP genes, beginning in the adaxial cells and progressing in the developing somites. At late stages of embryos, *myoD* mRNA is also detected in the fin bud and head muscles, and so are the three *MEF2* transcripts, consistent with our present observation on MSP genes. In addition, *MEF2A* and *MEF2C* are also expressed in the heart and they are the early markers of the cardiac cell lineage.

By combination of our present work on MSP gene expression, the temporal expressions of zebrafish muscle transcription factor genes and MSP genes are compared and shown in Figure 7A. In general, muscle transcription factor genes are expressed earlier than the MSP genes, consistent with their regulatory and upstream roles. But there are also exceptions. Aside from *desm*, which may also have a regulatory role and is expressed earlier than many transcription factor genes, the second earliest MSP gene, *tpma*, is expressed only after *myoD* and *MEF2D* genes, implying that *tpma* may be activated by fewer muscle transcription factors than other MSP genes. Thus, the activation of each MSP gene may require different myogenic transcription factors.

MSP genes and myogenic transcription factor genes are well characterized in avian and mammalian systems. There is also a sequential expression of these genes (Buckingham, 1992; Ontell et al., 1995). However, the order of expression is not always the same as that in zebrafish. Figure 7B summarizes the temporal expression of several MSP genes and myogenic genes in the mouse. It is interesting to note that several MSP genes are expressed even earlier than the myogenic determination gene, *myoD*. Comparing Figure 7A and B, we can observe several differences in the temporal activation of the MSP genes as well as the myogenic transcription factor genes between zebrafish and mice. For example, mouse *MLC2f* transcripts are detected

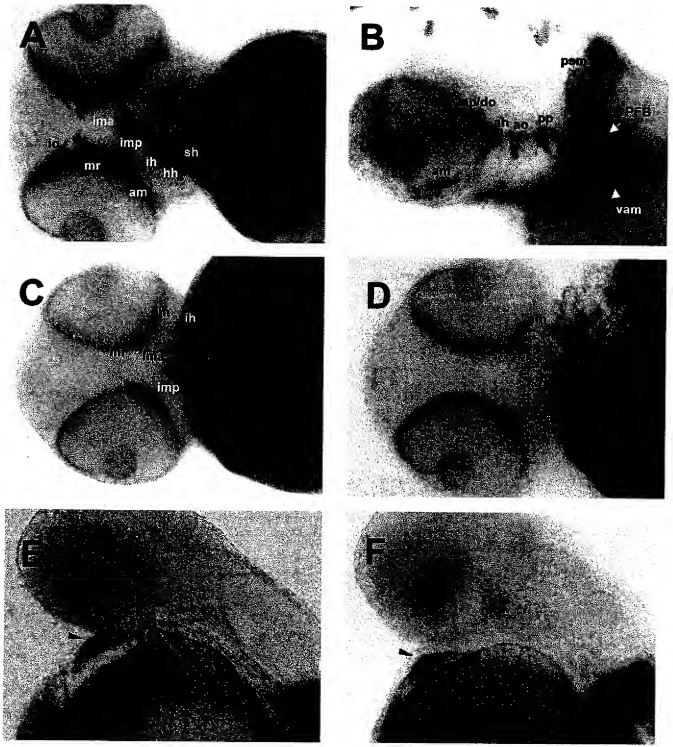

Fig. 6. Expression of MSP genes in late embryogenesis. **A:** Ventral view of the rostral part of a 72 hpf embryo hybridized with the *myl2* probe to show its expression in eye, jaw and gill muscles. **B:** Lateral view of the same embryo as shown in A with the anterior to the left. **C:** Expression of *desm* mRNA in a 60 hpf embryo (ventral view). **D:** Expression of *pvalb* mRNA in a 60 hpf embryo (ventral view). **E,F:** Lateral view of 48 hpf embryos hybridized with *desm* (E) and *ckm* (F) probes, respectively, to show their expression in the developing heart as indicated by an arrowhead. ah, adductor, hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyideus; ima, intermanibularis anterior; imp, intermandibularis posterior; io, inferior oblique; lap, levator arcus palatini, mr, medial rectus; PFB, pectoral fin bud; psm, presomite muscle; pp, protractor pectoralis; vam, vental abdominal muscle. The nomenclature of muscles is based on Schilling and Kimmel (1997).

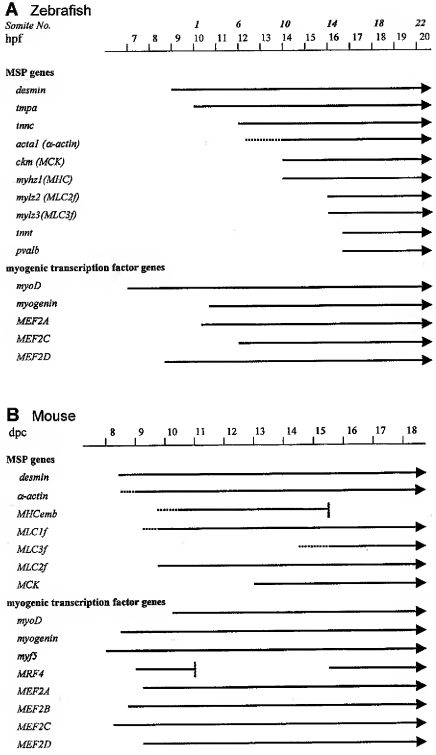

Fig. 7. Comparison of temporal appearance of MSP mRNAs with that of myogenic transcription factor mRNAs in zebrafish (**A**) and mice (**B**). Arrow lines indicate time span of expression for the embryonic stages that are defined by the number of formed somites and hours post fertilization (hpf) in zebrafish and days post coitum (dpc) in mice. Dotted line indicates weak expression. The zebrafish data were collected from this study for MSP mRNAs, from Weinberg et al. (1996) for *myoD* and myogenin mRNAs, and from Ticho et al. (1996) for *MEF2* mRNAs. The mouse data were compiled from Buckingham (1992), Faerman and Shani (1993), Li et al. (1993), Ontell et al. (1995), and Molkentin and Olson (1996). When the mouse gene names are different from those of zebrafish, the corresponding names are indicated in parenthesis in A.

prior to the activation of *myoD* gene and serves as an early marker for muscle differentiation. However, zebrafish *mylz2* (*MLC2f*) transcripts are detected much later than *myoD* gene, and serve as an intermediate marker for muscle differentiation. It is also worth noting that the zebrafish genome might have been duplicated compared to mammalian genomes (Amores et al., 1998; Postlethwait et al., 1998). Thus, there may be an additional othologous gene in zebrafish that is expressed earlier, at a time equivalent to the expression of the mouse ortholog.

Why different MSP genes are activated at different times is still not clear. Recent studies indicate that at least some of the MSP genes are cooperatively activated by MEF2 and myogenic bHLH transcription factors (Molkentin et al., 1995; Molkentin and Olson, 1996). Each myogenic regulatory gene, in turn, responds differently to external signals, as indicated by the fact that each of these myogenic transcription factor genes has a distinct pattern of expression (Buckingham, 1992). Because the MSP genes are the direct targets of the myogenic transcription factors, the differential expression of the former may result from the differential expression of the latter.

In addition to the temporal divergence, Ticho et al. (1996) found that there is also species-specific difference in the tissue distribution of *MEF2* genes among zebrafish, mouse, and *Xenopus*. For example, in zebrafish, no *MEF2D* transcript is detected in the heart, while *MEF2D* are expressed in cardiac muscles of both *Xenopus* and mouse. Similarly, while Faerman and Shani (1993) observed a transient expression of mouse *MLC2f* transcripts in the cardiomyocytes, no zebrafish *mylz2* (*MLC2f*) expression could be detected at any stage in the heart (Xu et al., 1999; this study).

Thus, despite the extensive structural and functional conservation of the muscle proteins among different vertebrate species, their transcriptional regulation in early development is divergent both in timing and tissue restriction. Ticho et al. (1996) suggest that the evolution of the genes that encode myogenic transcription factors in vertebrate genome must have preceded the evolutionary radiation of fish and mammals, but the different regulatory programs that specify the activation of these genes would appear to have evolved later. Because most of the MSP genes are likely the direct targets of the myogenic transcription factors, their expression appears to be also species-specific. Our data are consistent with this notion.

EXPERIMENTAL PROCEDURES

Zebrafish and Embryos

Zebrafish were purchased from a local fish farm and maintained in our aquarium. Embryos were collected after setting a photoperiod consisting of 14 hr of light and 10 hr of dark. The developmental stages were presented as hours postfertilization (hpf) at 28.5°C, based on Kimmel et al. (1995). For in situ hybridization, the number of somites was counted for every embryo during somitogenesis stages to ascertain the accurate developmental stages prior to fixation in 4% paraformaldehyde solution.

cDNA Clones and Sequence Analysis

Nine MSP cDNA clones were selected from our EST clones, which were isolated from either an zebrafish embryonic or an adult cDNA libraries (Gong et al., 1997). These clones are: E442 (*acta1*), E371 (*tpma*), A354 (*tnnc*), E134 (*tnnt*), E68 (*myhz1*), E72 (*mylz2*), E94 (*mylz3*), E146 (*ckm*), and E465 (*pvalb*). Clones derived from the embryonic or adult library were designated with E and A respectively. A full-length *desm* cDNA clone, which was previously isolated by screening the same zebrafish embryonic library using a PCR fragment (Loh et al., 1999) was also included in the study. These clones were sequenced from both ends by an automated sequencing machine ABI 377 (Perkin Elmer) using the ABI Prism dRhodamine Termination, Cycle Sequencing Ready Reaction kit.

Northern Blot Hybridization

Total RNA was isolated from various tissues of adult fish and from embryos of different developmental stages using TRIzol reagent (Gibco BRL). The RNA (10 μg) was fractionated on 1.2% formaldehyde-agarose gels and transferred to GeneScreen membranes (DuPont-New England Nuclear) as previously described (Gong, 1992). The blots were prehybridized at 42°C in a hybridization buffer [50% formamide, 5 × Denhardt's solution, 4 × SET (1 × SET = 0.15 NaCl, 1 mM EDTA, 20 mM Tris, pH 7.8), 0.2% NaPPi, 25 mM phosphate buffer, 250 μg/ml calf thymus DNA, and 0.5% SDS). Hybridization with a ^{32}P-labeled cDNA probe was performed in the same hybridization buffer at 42°C overnight. Membranes were washed first with 2 × SET/0.1% SDS and finally with 0.2 × SET/0.1% SDS at 65°C and exposed to X-ray film for autoradiography. Probes were labeled by the Random Primers DNA Labeling System (Gibco, BRL). The full-length inserts from the selected cDNA clones were used as templates for probe labeling except for *acta1* clone. To avoid its cross-hybridization with the β-*actin* mRNA, a 3′-UTR probe (~120 bp) starting immediately from the termination codon, was generated by PCR.

Whole-mount In Situ Hybridization

Whole-mount in situ hybridization using a digoxigenin (DIG)-labeled riboprobe was carried out essentially as reported by Korzh et al. (1998). The plasmid DNA was linearized with Bam HI, followed by in vivo transcription reactions with T7 RNA polymerase for the antisense RNA probe. For *acta1* expression, only the 3′-UTR was used as probe, similar to that in Northern blot hybridization. Some of the stained embryos were embedded in 1.5% sucrose/agarose and sectioned on a cryostat (15 μm). Two-color in situ hybridization was

performed according to Hauptmann and Gerster (1994).

ACKNOWLEDGMENTS

We thank Dr. V. Korzh for critical comments on the manuscript and for providing the *myoD* cDNA clone. We thank Ms. Yaling Guo for assistance in DNA sequencing. This work was supported by an NUS academic research grant to Z.G. Y.X. and X.W. were supported by an NUS postgraduate scholarship.

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EXHIBIT 11

Gong Zhiyuan

From: Liu Lei
Sent: Friday, July 24, 1998 1:33 PM
To: SCI Teaching Staff
Cc: Tio Gaik Hong
Subject: FW: PDF for FY1998--2000

Dear Faculty Staff,

Post-Doctoral Fellows (PDF) can be hired to serve the advance research manpower needs of existing projects in which equipment and consumables are already available.

If you would like to hire a PDF for your project (no vacancy for Post-Master Fellows (PMF) at the moment), please write in through the Head of Department to Dr Tan Eng Chye, Sub-Dean, Faculty of Science.

In your application, please include the following information:

- An abstract of the project;
- Progress to date;
- Achievement/deliverables;
- Justification for the need of PDF; and
- Potential candidates, if any.

Your write-up should not exceed 5 pages.

Each department can set its own time-table for submission. All applications should however reach the Dean's Office by **15 August 1998**.

If you have any queries, please contact me at extension 8300.

Regards,
Jacqueline Liu
Dean's Office

-----Original Message-----
From: Liu Lei
Sent: Friday, July 24, 1998 9:53 AM
To: SCI Teaching Staff; SCI Dean's Office SCIOff
Subject: RE: PDF for FY1998--2000

Dear All,

The Policies/Guidelines/Terms & Conditions for PDF and PMF are available on the Science website:
http://www.science.nus.edu.sg/Research/Postgrad/pdfpolicy.html
http://www.science.nus.edu.sg/Research/Postgrad/pmfpolicy.html

To: Dr. Tan Eng Chye,
Dean's Office
Faculty of Science

Through: Heads of DBS

RE: NSTB Postdoctoral Fellowship

We wish to apply for an NSTB postdoctoral position for our on-going Research Project RP960315, entitled **"Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP)"**

Principal Investigator: Dr. Gong Zhiyuan
Co-investigator: Prof. Lam Toong Jin

Abstract

Original abstract of the project: Ornamental fish is an important export industry in Singapore. In the present grant application, we propose to use a modern transgenic technique to generate novel varieties of ornamental fish by incorporation and expression of a jellyfish gene coding for green fluorescent protein (GFP). During the course of this work, a rapid cDNA clone tagging approach, or sequencing randomly selected clones by single run sequencing reactions, will be used to isolate and identify zebrafish genes in bulk. Interesting promoters will be isolated based on the sequence information from these tagged cDNA clones and characterized by transient expression in transgenic zebrafish. Useful promoters will be selected to generate stable lines of GFP transgenic zebrafish. The initial phase of this research is to focus on the following 5 patterns of GFP expression in transgenic zebrafish: ubiquitous expression, muscle specific expression, skin specific expression, heat inducible expression and heavy metal inducible expression.

So far, we have successfully isolated over three hundred non-redundant zebrafish genes and six gene promoters including skin specific, muscle specific, ubiquitous, heat-shock inducible and heavy-metal inducible gene promoters. By selection of proper gene promoters, we have successfully generated skin fluorescent, muscle fluorescent and uniformly fluorescent transgenic zebrafish. All these fish show specific patterns of green fluorescence. In future, we will also plan to develop multi-color fluorescent transgenic fish. In the meantime, we will also plan to use fluorescent transgenic fish to develop biosensor systems for monitoring environmental pollution. Efforts will also be made to isolate more gene promoters for generation of more varieties of fluorescent transgenic zebrafish. Other fish species will also be tested with the gene constructs developped from zebrafish. The flurescent transgenic fish will be commercialized and pattened together with the transgenic gene constracts and gene promoters.

Progress to date:

1. So far, we have isolated a few hundred zebrafish genes under this project and these genes encode a wide range of proteins located in all cellular compartments and expressed in a wide variety of tissues. Thus, these cloned genes provide a rich resource for developmental analysis and for isolation of gene promoters.

2. We have developed a rapid method to isolate gene promoters and so far six gene promoters have been isolated: one is from a cytokeratin gene for skin specificity; three for muscle specificity from a myosin light chain 2 gene and two muscle creatine kinase genes; one from small heat shock protein gene for inducibility by heat shock, heavy metal and stress; and one from acidic ribosomal protein P0 gene for ubiquitous expression.

3. We have demonstrated that the skin specific promoter and the muscle specific promoter can direct GFP expression correctly in respective tissues by transient transgenic assay and the ubiquitous gene promoter also direct a ubiquitous expression of GFP.

4. Stable line of GFP transgenic fish are being developed.

Future Work:

1. We will introduce the heat shock gene promoter to test the inducibility by heat shock and heavy metals. A long term objective of the work is to develop a biosensor system for monitoring environmental pollution such as heavy metals and toxic chemicals which may stress the fish to activate the heat chock gene promoters which in turn drives the expression of the green fluorescence gene. Similarly, a hormone inducible promoter such as the one from a vitellogenin gene will also be isolated to develop a different biosensor system for monitoring pollution from estrogen and its derivatives.

2. For all transgenic fish, we will demonstrate germ line transformation and ensure the stable inheritance and expression of GFP transgene. These will need a few years to complete as each generation will take about half year under our laboratory condition and for observation of stable transgene transmission, we should test for at least three generations.

3. More tissue specific gene promoters will be isolated to generate more varieties of transgenic fish, for example, eye specific, fin specific, liver specific, or heart specific etc.

4. Technique advances make other colorful fluorescence proteins available and these include blue fluorescent protein (BFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP). These present us the opportunity to generate multiple color fluorescent transgenic fish. With the wide range of tissue specific promoters, we will be able to generate colorful chimerical transgenic fish, for example, green skin/blue muscle/yellow eyes and many other combinations.

5. Generation of these multiple fluorescence color will need extensive breeding for several generations and thus need at least three more years to complete. After we complete the first research project by August 1999, we will seek another grant to continue the promising fluorescent transgenic fish work.

6. We will also test all successful zebrafish gene constructs in other fish species to investigate their suitability as universal constructs for gene transfer in all fish species.

7. Development of stable GFP transgenic lines should also be valuable for many other studies. Since detection of GFP is a non-invasive approach and expressed GFP can be conveniently observed by epifluorescence microscopy, GFP transgenic lines will facilitate the study of cell lineage and cell migration if GFP is expressed in a tissue specific manner. GFP transgenic zebrafish, particularly under a ubiquitous promoter, will also be valuable for cell transplantation and nuclear transplantation experiments because GFP and GFP transgene can be conveniently used as cellular and genetic markers for fish cloning.

Achievements (Dr. Gong's lab):

Under the present research project, we have isolated over three hundred fish genes from over 1,300 randomly selected cDNA clones. Six gene promoters have been isolated and characterized. Skin fluorescent, muscle fluorescent and uniformly fluorescent transgenic fish have been developed for the founder generation.

Because of this work, our laboratory has been a world leading laboratory for fish gene cloning and is emerging as a world leader for fish gene promoters under the current project. Our work has won wide recognition internationally, as evident by the following facts:

1. Invitations to international conferences/workshops/scientific program (Dr. Gong):

a. **Selected speaker** "Rapid identification and isolation of zebrafish genes by cDNA clone tagging" Zebrafish Development & Genetics 1996, Cold Spring Harbor Laboratory, U.S.A. April 1996.

b. **Keynote Speaker**, "Transgenic fish and marine biotechnology" Asia-Pacific Conference on Science and Management of Coastal Environment, Hong Kong, June 1996.

c. **Invited Speaker**, "Sequence tag project in the zebrafish" in Current Advances in Defining the Zebrafish Genome, Boston, MA, U.S.A. Feb. 1997.

d. **Session Chair and Invited Speaker,** "Zebrafish neuroD, a potential upstream gene of the neuroendocrine transcription factor Isl-1". 2nd IUBS Toronto Symposium "Advances in the Molecular Endocrinology of Fish" May 16-19, 1997, Toronto, Canada.

e. **Advisory Board member**, IUBS-RBA (International Union of Biological Sciences-Reproductive Biology in Aquaculture) Program. Since May 1997.

f. **Session Chair and Invited Speaker,** "Massive cloning of zebrafish genes and their applications". 7th SCBA International Symposium. July 6-11, 1997, Toronto, Canada.

g. **Invited Speaker,** "Application of transgenic techniques in fish and shrimp diseases" UNESCO workshop on shrimp disease, Oct. 9-14, 1998, Qingdao, China.

2. Our gene cloning work has received worldwide attention. So far, we have received over 70 requests (incomplete statistics) for cloned fish genes and libraries from USA, Canada, UK, France, Germany, Japan, Russia, China, Korea, New Zealand, Ireland, Hungary, Hong Kong and Singapore.

3. Dr. Gong has been invited to contribute a chapter to describe the method we used for rapid isolation and identification of fish cDNA clones by an authoritative book series, *Methods in Cell Biology* (Academic Press).

4. Dr. Gong has been invited to deposit the tagged zebrafish cDNA clones to ATCC (American Type Culture Collection), the world largest non-profit institute for a centralized scientific community resource repository.

5. Our successful generation of fluorescence transgenic fish has attracted intensive attentions from the media. It was first covered by an article in a prominent Japanese newspaper *Nikkei*, followed by local newspapers, *Straits Times* and *Lian He Wan Bao*. A television interview (AM Singapore) will be followed.

Deliverables:

1. Currently three major research paper has been submitted to or in preparation for top-notch international refereed journals. After completion of the project, there should be many more to come. Our track record indicates that we have the ability to publish more papers as in the past three years since Dr. Gong joined NUS, Dr. Gong's group has published 12 research papers in prestigious international refereed journals and two invited articles/book chapters. In addition, currently 8 manuscripts have been submitted or are in preparation and the complete list of these papers is attached in Appendix A.

2. Directly under this project, one lab technologist and two postgraduate students have been trained.

3. There are two major applications of transgenic fluorescent fish: one is generation of a wide variety of colorful fluorescent fish for ornamental fish industry and the other the development of a biosensor system for monitoring environmental pollution such as heavy metals and organic chemicals. Because of these obvious commercial potentials, we plan to patent the followings at late stage of the project:
1) fish gene promoters
2) transgenic DNA constructs
3) transgenic florescent fish

Justification:

1. As our laboratory is emerging as a world leader in fish molecular biology, it is important to recruit a postdoctoral fellow (PDF) at this stage to enhance our competitive ability in the first league.

2. A PDF will be also important to help the professor to supervise lab technologists and graduate students in a big laboratory. Currently the laboratory consists of 2 lab technologists, 8 graduate students and 2 Honors students. From time to time, a few undergraduate students will join the lab for short term projects.

3. Our track record indicates that we can make good use of manpower. Essentially everyone who joins the lab can make rapid progress because of our strong and ever-growing research program. We have excellent molecular resources for all different kinds of research projects in fish biology and essentially all the-state-of-the-art techniques in molecular biology. One of the best members in the lab, Liao Ji, has published or will publish at least 8 research papers during her half year as a lab technologist and one year of Master candidature.

4. The success of the fluorescent transgenic fish project is largely dependent on the person who carries out the gene delivery experiment. This experiment requires a highly specialized skill to perform microinjection of DNA into a single cell under a microscope. This skill needs a long time of training and unfortunately not everyone can be trained for this skill. The postdoctoral candidate, Mr. Ju Bensheng, is one of the few workers in Singapore who have such a skill and he is currently employed as a lab technologist (grade B) under the present project. However, he has a Master degree from China, has completed a Ph.D program in NUS and will have his Ph.D defense on August 14, 1998. It is not possible to keep him at the lab technologist position, as he is applying for postdoctoral positions overseas vigorously. Dr. Gong has talked to him about the NSTB Postdoctoral Fellowship and he is willing to accept the Fellowship to continue the transgenic fish project. To retain Mr. Ju in the project is crucial to the final success of transgenic fluorescence fish as he has single-handedly set up the transgenic system in our laboratory, and now it is at the stage to harvest the fruits after many years of fundamental research.

5. Although the current research project (RP960315) will be completed by July 1999, we will seek another research grant to continue the promising transgenic fish work. As proposed in the Future Work, we plan to extend this project for generation of multi-color fluorescent transgenic fish and development of biosensor systems to monitor environmental pollution. These works will require tremendous manpower. We plan to keep Mr. Ju at a postdoctoral position to supervise the overall transgenic fish project and to perform microinjection experiments. As dozens of transgenic fish lines will be generated throughout the project, a full time lab technologist will also be needed to maintain these transgenic lines including breeding for multiple generations. Hopefully, within two years, Mr. Ju will help to train a lab technologist to master the microinjection technique and eventually the latter will take over Mr. Ju's duties. In the meantime, one or two postgraduate students will be recruited to isolate and characterize more gene promoters.

6. Currently, Dr. Gong has another research grant (RP3972393) on zebrafish developmental biology which will run until March of 2001. Therefore, we should have sufficient consumables provision for the PDF's research. In addition, Dr. Gong's lab is reasonably equipped with all necessary instruments for carrying out the work. Other essential equipment such as fluorescence microscope, cofocal microscope, and needle puller etc. can be accessed within our department. Thus, there will be no extra consumables and equipment requested for the new PDF in the next two years.

7. Because of our strong expertise in molecular biology and gene cloning, the postdoctoral candidate will be trained as an expert for these techniques, which are much needed in the current and future job market. Therefore, the candidate should be able to find a position in research institutes and biotech companies in Singapore after two years of postdoctoral research here.

Potential Candidate:

Mr. Ju Bensheng
Date of birth: Oct. 14, 1966
Nationality P. R. CHINA
Marital Status: Married

Employment
July 1992- May 1994 Assistant lecturer in Fisheries College, Ocean University of Qingdao (OUQ), P.R.CHINA
Dec. 1997-present. Lab. Technologist in School of Biological Sciences, National University of Singapore

Education

June 1994- Dec. 1997	Ph.D. candidate in School of Biological Sciences, National University of Singapore Thesis submitted and thesis viva to be held on Aug. 14, 1998.
July 1992-June 1994	M. Sc. Fisheries College, OUQ
July 1989-July 1992	B. Sc Fisheries College, OUQ

Appendix A: List of Publications in NUS

1) Tokumoto, M., Z. Gong, T. Tsubokawa, C.L. Hew, K. Uyemura, Y. Hotta, and H. Okamoto (1995) Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel Islet homologs in embryonic zebrafish. *Dev. Biol.* **171**: 578-589.
2) Gong, Z., K.V. Ewart, Z. Hu, G.L. Fletcher, and C.L. Hew (1996) Skin antifreeze protein genes of the winter flounder, Pleuronectes americans, encode distinct and active polypeptides without the secretory signal and prosequences. *J. Biol. Chem.* **271**: 4106-4112.
3) Liao, J., J. He and Z. Gong (1997) An abundant zebrafish cDNA clone encodes a ras-like protein which is expressed ubiquitously. *DNA Sequence* **7**:313-317.
4) Liao, J. and Z. Gong (1997) Sequencing of 3' cDNA clones using anchored oligo dT primers. *BioTechniques* **23**:368-370.
5) Gong, Z., T. Yan, J. Liao, S.E. Lee, J. He and C.L. Hew (1997) Rapid identification and isolation of zebrafish cDNA clones. *Gene* **201**:87-98.
6) He, J., Z. Yin, G. Xue, Z. Gong, T.J. Lam and Y.M. Sin, Production of goldfish against *Ichthyophthrius multifilis* by immunization with a recombinant vaccine (1997) *Aquaculture* **158**:1-10.
7) Liao, J., C.H. Chan and Z. Gong (1997) An alternative linker-mediated polymerase chain reaction method using a dideoxynucleotide to reduce amplification background. *Anal. Biochem.* **253**:137-139.
8) Lim, J.H., J. He, V. Korzh and Z. Gong (1998) A new splicing variant of a type III POU gene from zebrafish encodes a POU protein with a distinct C-terminal. *Biochim. Biophy. Acta* **1379**:253-256.
9) Postlethwait, J.H., Y.-L. Yan, M.A. Gates, S. Horne, A. Amores, A. Brownlie, A. Donovan, E.S. Egan, A. Force, Z. Gong, C. Goutel, A. Fritz, R. Kelsh, E. Knapik, E. Liao, B. Paw, D. Ransom, A. Singer, M. Thomson, T.S. Abduljabbar, P. Yelick, D. Beier, J.-S. Joly, D. Larhammar, F. Rosa, M. Westerfield, L.I. Zon, S. Johnson and W. Talbot (1998) Vertebrate genome evolution and the zebrafish map. *Nature Genetics* **18**:345-349.
10) Miao, M., S.-L. Chan, C.L. Hew and Z. Gong (1998) The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitous enhancer lacking a functional C/EBPα binding motif. *FEBS Letters* **426**: 121-125.
11) Fletcher, G.L., S.V. Goddard, P.L. Davies, Z. Gong, K.V. Ewart and C.L. Hew (1998) New insights into fish antifreeze proteins: physiological significance and molecular regulation. In: Cold Ocean Physiology (H.O. Portner and R.C. Playle. eds.) pp.239-265.
12) Gong, Z. (1998) Zebrafish expressed sequence tags and their applications. Submitted to *Methods Cell Biology* (zebrafish volume, invited article) **60**, In press.
13) Yan, T., and Z. Gong (1998) Assembly of a complete zebrafish mitochondrial 16S rRNA gene from overlapping expressed sequence tags. *DNA Sequence* In press.
14) Korzh, V., I. Sleptsova, J. Liao, J. He, and Z. Gong (1998) Expression of zebrafish bHLH genes ngr1 and neuroD defines stages of an early neural differentiation. *Dev. Dynamics*. In press.

Manuscript submitted or in preparation:

15) Garg, R.R., L. Bally-Cuif, S.E. Lee, Z. Gong, X. Ni, C.L. Hew, and C. Peng. Molecular cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and expression of ActRIIB mRNA in embryos and adult. Submitted to *Endocrinology*.

16) Xu, Y., J. He, H.L. Tian, C.H. Chan, J. Liao, T. Yan, T.J. Lam and Z. Gong. Fast skeletal muscle specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. Submitted to *DNA Cell Biol.*

17) Ju, B., Y. Xu, J. He, J. Liao, T. Yan, T.J. Lam and Z. Gong. Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under homologous zebrafish gene promoters. To be submitted to *Dev Biol.*

18) Dheen, T., I. Sleptova-Friedrich, Y. Xu, M. Clark, H. Lehrach, Z. Gong and V. Korzh. Zebrafish tbx2 plays a role in formation of the midline structures. To be submitted to *Development.*

19) Wang, H., T.Tan, T. Yan and Z. Gong. A zebrfish cDNA clone encodes a novel vitellogenin without a phosvitin domain and represents a primitive vertebrate vitellogenin gene. In preparation.

20) Wang, H. and Z. Gong. Both egg proteins ZP2 and ZP3 mRNAs are synthesized specifically in liver in zebrafish. In preparation.

21) He, J., V. Korzh, Y.M. Sin, T.J. Lam and Z. Gong. A zebrafish vimentin gene is specifically expressed in a subset of neurons in central and peripheral nervous systems. In preparation.

22) Gong, Z., J. Liao, J. He, T. Yan, V. Korzh. Characterization of three novel zebrafish cDNA clones encoding neuroD-like basic helix-loop-helix transcription factors and their expression in developing nervous system. In preparation.

FACSIMILE TRANSMISSION
NO. OF PAGES: 3

Founded 1905

THE NATIONAL UNIVERSITY *of* SINGAPORE

URGENT

Ref:

Dean's Office
Faculty of Science

27 August 1998

To:

Biological Sc.	[redacted] Zhiyuan	Compul. Sc.	Dr Chen Yu Zong
	A/P Lim Tit Meng	Materials Science	Dr Ding Jun
	Dr Ge Ruowen		A/P Xu Gu
Chemistry	Dr Xu Guo Qin		Dr Li Yi
	A/P Andy Hor	Physics	Dr Andrew Wee
	Dr Wong Ming Wah		A/P Lai Choy Heng
	Dr Chin Wee Shong		A/P Frank Watt
Compul. Sc.	Asst/Prof Wei Guowei		Dr Shen Ze Xiang

PDF Position FY 1998-2000

I am pleased to inform you that Dean has approved PDF positions for the projects given in **Table A**. Projects that have been given provisional acceptance, subject to availability of places and good candidates are shown in **Table B**.

I would like to meet the Principal Investigator of all these projects on **31 August 1998** at **4 p.m.** in the **Dean's Office Conference Room, Level 9**. Please call Ms Gaik Hong at ext. 3333 to inform her whether you are able to attend the meeting.

Thank you.

NATTANEC@

A/P Tan Eng Chye
Sub-Dean
Faculty of Science

age 33yr
Mid-Oct deadline

cc: Head, Department of Biological Sciences
Head, Department of Chemistry
Head, Department of Computational Science
Head, Department of Materials Science
Head, Department of Physics

Table A: **Approved**

Dept	Project Title	Principal Investigator
DBS	Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP)	Dr Gong Zhiyuan
DBS (Bioscience)	Bioactive compound research	A/P Lim Tit Meng
Chemistry	In-situ studies on the interaction of metal atoms with aromatic molecules	Dr Xu Guo Qin
Chemistry	Chemical behavior and technological applications of fullerenes, nanotubes and nano-particles	A/P Andy Hor
Chemistry	Molecular modeling of chemical synthesis of nanostructured films	Dr Wong Ming Wah
Comput. Sc	Wavelet approach to linear and nonlinear dynamical problems	Asst/Prof Wei Guowei
Mat. Sc.	Novel Magnetic Materials	Dr Ding Jun
Mat. Sc.	Improved organic light-emitting devices	A/P Xu Gu
Physics	Upgrading of Surface Science facility for SIMS studies of advanced materials	Dr Andrew Wee
Physics	Synchronization and control of chaotic dynamical systems: applications in secure communications	A/P Lai Choy Heng
Physics	Proton micromachining: manufacture of microdevices	A/P Frank Watt
Physics	Development of an ultra-high spatial resolution scanning Raman microscope and its applications in semiconductor device research and characterisation	Dr Shen Ze Xiang

EXHIBIT 12

Date of injection	Construct	No injected	50% epiboly S	50% epiboly E	12 hrs S	12 hrs E	24 hrs S	24 hrs E	48 hrs S	48 hrs E	Tissue Specificity of Expression: Skin	Muscle	Eye	Blood	Nerve	Notocord	REMAI
12/5/99	MLC 200bp (L)	75	60	0	56	0	50	8	48	23	2	28 (3)	1	0	2	2	++
13/5/99	MLC 1Kb (L?)	60	52	0	50		46	2	46	26	3	26	0	0	2	0	+++
14/5/99	200bp+en enhancer?	30	24	0	20	0	20	0	17	6	3 (yolk)	4			2	1	(1) ++
15/5/99	100bp+enhancer	20	16	0	14	0	14	0	14	2	/	2		/	/	/	+
15/5/99	200bp+en	30	24	0	23	0	21	14			10 7					2	++ (2)
..	..	..	..		..		..	..	21					1 (heart muscle)			
19/5/99	0.8Kb ARP+en	60	48	-	45	44	45	12	45	10	Strong expression 12hpf. weak at 24hpf						} due to enhancer
20/5/99	ARP0.8Kb	48	40	-	36	8	32	6	20	4	Weak throughout						
18/5/99	MLC 1.5Kb (L)	60	52	0	48	0	36	14	36	30	3	30	2	2 (heart muscle)	0	0	(3) +++ (4) +
21/5/99	MLC 500bp (L)	58	48	0	44	0	38	12	32	23	4	23		0	2	0	(3) +
22/5/99	MLC 500bp (L)	76	60	0	54	0	50	12	48	32	5	32	-	2 (heart muscle)	2	2	(5) +
14/5/99	MLC x CKP F1								Exp (+) 32/280		CKP(+) 14 MLC (+) 22 CKP/MLC (+) 2						

+++ very strong
++ strong
+ expression in scattered fibres

	Date of injection	Construct	No injected	50% epiboly S	50% epiboly E	12 hrs S	12 hrs E	24 hrs S	24 hrs E	48 hrs S	48 hrs E	Skin	Muscle	Eye	Blood	Nerve	Notocord	REMARK
	06/09/98	ARP 0.8kb	40	32						14 (35) (3 normal)	2	/	/	/	/	2	/	weak exp
	..	ARP 2.2kb	60	45	36					19 (37) (7 normal)	13	13	10	1	1	13	12	Strong ex
	..	MCK 1.5kb	20	16	0					12 (60)	2	/	2	/	/	/	/	weak
2kb ✓	..	MLC 2.2kb	68	(?)	0					42 (62) (32 normal)	6	/	6	/	/	/	/	strong
2kb	07/09/98	MLC 2.2kb (L)	40	28	0	26	0	22	6	22	12	12	12	/	3 (heart)	/	/	(4) (4) +++ ++
X	12/09/98	ARP 2.2 (L)	40	32	17	27	19	21	16	18	15	11	5	2	/	15	9	weak
X	07/10/98	ARP (2.2) L	58	20	14			18	15			/						1 strong
2kb ✓	05/11/98	MLC 2kb (L)	38	38				32	8	31	29	2	29	2	7 (heart muscle)	72 hrs	(+++) (18)	cell inject
same batch 2kb	..	.. (L)	32	32				30	13 (41)	29		1	25	1	3 (heart muscle)		/	1 cell injec ++13 ++10
✓	06/11/98	MLC 1000bp (L)	54	52				48	10	46	8	2	6	barely observable			4	weak
	06/11/98	MLC 1000bp (L)	28	28				28	5	20	2	Expression barely observable					2	weak
	10/11/98	MLC 1kb (L)	50	32				30 (10k)	6	30	19	3	19	/			/	1 strong (+) the rest we
	11/11/98	MLC 1.5kb (L)	97	84	0			66	10	63	15	2 weak and non-muscle specific (m.s)			2 (heart muscle)			36(+)
2kb ✓	22/12/98	MLC 2kb (L)	76	72	0			60	2 (weak)	60	22	3 5	22 53	1 2	2 (heart muscle) 4 (heart muscle)		/	
2kb ✓	08/12/98	.. (Linear)	88	74	0			68	8 (weak)	66	53	from (+) to (++) 4 in heart.						
	6/2/1999	CKP (L)	52	4				46	46									

S - survival
E - Expression

Date of injection	Construct	No injected	50% epiboly S	50% epiboly E	12 hrs S	12 hrs E	24 hrs S	24 hrs E	48 hrs S	48 hrs E	Tissue Specificity of Expression Skin Muscle Eye Blood Nerve Notocord	REMARI
18/10/98	100ng/ml ARP 1.3Kb(c)	20	20	0			19	0	19	0		
19/10/98	100ng/ml ARP1.1Kb	20	20	4			18	16	18	16	8 12 6 2 16 4	
23/10/98	MLC1.1Kb	50	42	0			30	1	26	5		
"	MLC1.0Kb	61	38	0			16	0	14	1		
25/10/98	MLC0.9Kb	60	20	0			12	4	8	4		

EXHIBIT 13

RESEARCH PROPOSAL SUBMISSION CHECKLIST

Please complete this checklist for every research proposal to be submitted to the Faculty Research Committee, Faculty of Science.

Ensure that you have included all the necessary information in your application and have arranged the documents in the sequence stated below.

Incomplete applications will not be processed.

(Please tick)

1. [√] Information on existing projects (no. of projects, titles of projects, start and end dates, balance of funds).
2. [√] Application Form - BUR/RG1.
3. [√] Appendix to BUR/RG1 - Case for Support.
4. [√] Track record of PI -
   e.g. RPXXXXXX Title of Project
   Funding:
   Research Output:
   (a) Publications (to be listed in standard reference format);
   (b) Student Theses;
   (c) Manpower trained; and
   (d) Patents (give some details).
5. [√] Brief CV of PI and collaborators with a list of 10 relevant publications.
6. [ ] List of potential referees (for projects with total value above $180,000 or total equipment value above $100,000).
7. [√] Brief CV of Research Fellow(s) to be employed.
8. [√] Quotations for equipment requested.

Submitted by:

Gong Zhiyuan / DBS		Feb. 1, 1999
Name/Department of PI	Signature of PI	Date

Information on existing projects -- Dr. Gong Zhiyuan

1. **RP960315, Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP).**

Start date: 07/96
End date: 07/99
Funding: $114,500
Balance by 12/98: $ 51,463.58

2. **RP3972393, 1998-2001: Molecular dissection of neurogenic pathway in zebrafish.**

Start date: 02/98
End date: 02/2001
Funding: $249,457
Balance by 12/98: $ 204,635.45

Multi-Disciplinary Research : / No + (Delete where appropriate)

NATIONAL UNIVERSITY OF SINGAPORE
ACADEMIC RESEARCH FUND
APPLICATION FOR A RESEARCH GRANT

TO: THE FACULTY RESEARCH COMMITTEE (PROJECT VALUE < $250,000)

1 PRINCIPAL INVESTIGATOR Name: Dr. Gong Zhiyuan Employee number: 11259H Appointment: Senior Lecturer Department: Biological Sciences Tel: 874-2860 Fax: 779-4801	Attach 1-page C.V. of Principal Investigator, giving an outline of education and work experience, track records in managing research projects and list (not more than ten) selected relevant and top publications.
Previous grants from Academic Research Fund: RP950304 ($209,435, 07/95-03/98) RP950328 ($70,300, 07/95-07/97) RP960315 ($114,500, 09/96-09/99) RP3972393 ($249,457, 02/98-02/2001)	To state the date/amount of previous grants. If the grant includes equipment, indicate the equipment purchased and the current / proposed usage of the equipment after project ended/ends.
2 * COLLABORATOR(S)/OTHER KEY TEAM MEMBERS Name: Professor Lam Toong Jin Employee number: 00706G Appointment: Professor and Head Department: Biological Sciences Tel: 874-2692 Fax: 779-2486	To provide details for each collaborator/key team member: Attach 1-page C.V. of each member, giving an outline of education and work experience, track records in managing research projects and the number of international journal and conference papers.
Previous grants from Academic Research Fund:	To state the date/amount of previous grants.

* Please use a separate sheet if there is insufficient space and attach it to this form.
\+ Defined as any research that requires input from staff in a different department or staff belonging to other disciplines from other institutions outside the University.

3 TITLE OF RESEARCH PROJECT Production of fluorescent transgenic ornamental fish	concise.
4 ABSTRACT Ornamental fish is an important export industry in Singapore. In the present grant application, we propose to continue our previous work on generation of fluorescent zebrafish by transgenic expression of green fluorescent protein (GFP). In the past two and half years, we have transferred the jellyfish GFP gene into the zebrafish under several different homologous zebrafish gene promoters and these transgenic fish displayed skin fluorescence, muscle fluorescence or ubiquitous fluorescence. In the present proposal, we will maintain and develop stable transgenic lines for these fluorescent zebrafish. We will also make use of the gene constructs developed from the zebrafish to produce other fluorescent transgenic ornamental fish of high market value, including medaka, goldfish, koi carp and glass catfish. In the meantime, we will also develop multi-color fluorescent transgenic fish by introducing several artificial GFP variant genes including BFP (blue fluorescent protein), YFP (yellow fluorescent protein) and CFP (cyan fluorescent protein) genes. By using different tissue-specific promoters, combinations of multiple colors in different tissues will be produced, e.g. green skin, blue muscle and yellow eyes, or other combinations. A bioseasor system will be explored by using a heavy metal-inducible gene promoter to monitor aquatic environmental pollution.	In about 200 words, describe the project in the context of previous work done or in progress at the University or at other institutions, and explain the uniqueness of this approach.
5 LIST MAIN OBJECTIVES IN ORDER OF PRIORITY 1. Development and maintanence of stable transgenic lines of green fluorescent transgenic zebrafish with different tissue specificities; 2. Development of multi-color fluorescent transgenic fish 3. Generation of stress-inducible GFP transgenic fish for a biosensor system; 4. Production of fluorescent transgenic medaka; 5. Production of fluorescent transgenic goldfish; 6. Production of fluorescent transgenic glass catfish; 7. Production of fluorescent transgenic koi carp. After completion of the project, there should be a few papers for publication in high profile international journals. Patents for each transgenic fish will be sought and fluorescent transgenic fish will be commercialized.	Describe the objectives clearly and succinctly, and highlight the deliverables upon project completion. Attach a self-contained case for support, consisting of no more than 6 A4 pages. Some assistance in preparing of this is given in Annex A.
6 POTENTIAL APPLICATIONS/EXPLOITATION The project concentrates on development of fluorescent ornamental fish, which will be marketable as a new category of exotic fish. The transgenic technique developed and fish gene resources explored in this study will also be applicable to other desirable traits with important economic implication, such as increase of growth rate, disease resistance and sex reversal etc.	State the likely applications of the work (technological, social, scientific, economic). Also explain any exploitation potential, and the follow-up arrangements that would be required.

7 COLLABORATIONS	Where appropriate, describe any collaborative arrangements, including arrangements for exploitation and protection of intellectual property.
Not Available.	

8 SUMMARY OF RESEARCH GRANT REQUESTED

Grant requested must cover the entire project life. Applicants should note that research grant, once approved, will not be increased.

	Year 1	Year 2	Year 3 *	Total ($)
Manpower	2,000	23,500	47,050	72,550
New equipment/facilities	5,250	0	0	5,250
Materials/consumables	27,600	24,600	24,600	76,800
Overseas Travel	0	0	0	0
Training/other misc. costs	1,200	1,200	1,200	3,600
Grand Total ($)	36,050	49,300	72,850	158,200

* Please use a separate sheet if project life is expected to exceed 3 years. The Total column should reflect the total grant required for the entire project life.

Please see Notes for Budget Preparation in Annex B for assistance in completing items 8.1 to 8.4.

8.1 MANPOWER COSTS (for additional staff only)

Please indicate an "E" against the number if it is a continuation of an existing appointment.
NS increments* - Please tick against staff grade if increments are to be given for National Service (NS). If the manpower required is a Research Fellow, please provide information on his/her qualifications and experience.

Manpower	Staff Grade	With NS*	Number		Annual Cost ($)			No. of Months on Project	Total Cost ($)
			Full Time	Part Time	Year 1	Year 2	Year 3		
Research Assistant			1			21,500	45,050	18	66,550
Technician/Jr Research Assistant									
Student Assistant									
Research Scholar									
Research Student				1	2,000	2,000	2,000	12	6,000

8.2 NEW EQUIPMENT/FACILITIES COSTS

Item Description	Unit Price ($)	Quantity	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
-20°C freezer	5,250	1	5,250			5,250
Grand Total ($)			5,250			5,250

Please append a list of existing equipment that will be used in the project. Equipment descriptions, costs and locations must be provided.

Item Description	Unit Price ($)	Quantity	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
molecular reagents	100	150	5,000	5,000	5,000	15,000
chemicals, glassware	100	150	5,000	5,000	5,000	15,000
radioisotopes	200	30	2,000	2,000	2,000	6,000
oligonucleotides	80	60	1,600	1,600	1,600	4,800
films and pictures			1,000	1,000	1,000	3,000
sequencing kit/software	1000	15	5,000	5,000	5,000	1,500
Fish, feed and tanks			8,000	5,000	5,000	18,000
Grand Total ($)			27,600	24,600	24,600	76,800

8.4 OVERSEAS TRAVEL

Item Description		Purpose	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
Country	No. of days					
Grand Total ($)						

8.5 TRAINING/OTHER MISCELLANEOUS COSTS

Item Description	Purpose	Year 1 ($)	Year 2 ($)	Year 3 * ($)	Total Cost ($)
Taxi fare	Live fish transportation	200	200	200	600
Miscellaneous	IDD, Fax, courier, patent search etc.	1,000	1,000	1,000	3,000
Grand Total ($)		1,200	1,200	1,200	3,600

* Please use a separate sheet if project life is expected to exceed 3 years. The Total column should reflect the total grant required for the entire project life.

9 OTHER SOURCES OF FUNDING	
Name and address of other funding parties: Contact name: Contact number:	
Type of organisation: (eg industry, commerce, research institutes, government, etc)	
Details of contribution: Cash: Equipment/materials: Staff secondment: Facilities: Others: **Total value of funding ($):**	

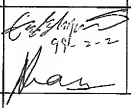
10 PROJECT IMPLEMENTATION SCHEDULE

Quarters/Research milestones	Year 1				Year 2				Year 3			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Objective 1	X	X	X	X	X	X	X	X	X	X	X	X
Objective 2					X	X	X	X	X	X	X	X
Objective 3	X	X	X	X	X	X	X	X	X	X	X	X
Objective 4		X	X	X	X	X	X	X	X	X	X	X
Objective 5				X	X	X	X	X	X	X	X	X
Objective 6						X	X	X	X	X	X	X
Objective 7								X	X	X	X	X

Estimated start date: 08/99	The start date is defined as the first date on which the project commits or incurs expenditure.
Estimated completion date: 08/2002	Researchers are reminded that a project, once approved, must start within 60 days of approval.

11 DECLARATION

We declare that the facts stated in this application and the accompanying information are true.

	Signatures and dates	
	Principal Investigator	Collaborating party (if any)
Applicant(s) : Dr. Gong Zhiyuan Professor Lam Toong Jin		
Endorsed by: (1) Head of Department (2) Chairman, Faculty Research Committee or Enterprise (3) Director of Research (4) Chairman, University Research Committee OR Chairman, Academic Research Fund Committee		

12 HEAD'S COMMENTS :

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

Signature Date

13 FACULTY RESEARCH COMMITTEE'S COMMENTS :
Please note that specific comments are required as indicated in Guidelines for Vetting Research Proposals.

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

If total project value is less than $250,000, please indicate if FRC approves/rejects the proposal:
Approve ☐ Reject ☐

Signature Date

14 DIRECTOR OF RESEARCH'S COMMENTS :

Please indicate your grading of the project:

A. ☐ B. ☐ C. ☐

Signature Date

I. PURPOSE

Singapore is the world largest exporter of ornamental fish with a revenue of about $100 million dollars per annum. To maintain the leadership in this competitive industry, it is necessary and crucial to continuously produce new varieties with novel shapes and color patterns. Traditional approaches to create new varieties are genetic breeding and selection, but these approaches are rather slow and unpredictable. The use of color dyes in many pet stores is either temporary or unsatisfactory. In the past two and half years, we have successfully generated fluorescent zebrafish by transgenic expression of a jellyfish green fluorescent protein (GFP) under different fish tissue-specific gene promoters and this approach provides a promissing way to generate new varieties of ornamental fish with distinct and predictable color patterns. In the present proposal, we will try to apply the same techniques to other more exotic ornamental fish species such as Japanese medaka, goldfish, koi carp and glass catfish. Meanwhile, multi-color fluorescent transgenic fish will be developed. The possibility of using a stress response promoter, such as a heavy metal-inducible promoter, to develop a biosensor system to monitor aquatic environmental pollution will also be explored. The transgenic technique developed and fish gene resources explored in this study will also be applicable to other desirable traits with important economic implication, such as increase of growth rate, disease resistance and sex reversal etc.

II. BACKGROUND

(i) Previous work

Transgenic technique involves transfer of a foreign gene into a host organism enabling the host to acquire a new and inheritable trait. The technique was first developed in mice at the beginning of 1980s by Gordon et al. (1980). They injected foreign DNA into fertilized eggs and found that some of the mice developed from the injected eggs retained the foreign DNA. Applying the same technique, Palmiter et al. (1982) have introduced a chimeric gene containing a rat growth hormone gene under a mouse heavy metal inducible gene promoter and generated the first batch of genetically engineered supermice, which are almost twice as large as non-transgenic siblings. This work has opened a promising avenues in using the transgenic approach to render transgenic animals new and beneficial traits for livestock husbandry and aquaculture.

In addition to stimulation of somatic growth for increasing the gross production of animal husbandry and aquaculture, the transgenic technique also has many other potential applications. First, transgenic animals can be used as a bioreactor to produce commercially useful compounds by expression of a foreign gene in milk or in blood. Many pharmaceutically useful protein factors have been expressed in this way. For example, the human α1-antitrypsin, which is commonly used to treat emphysema, has been expressed at a concentration as high as 35 mg/ml (10% of milk protein) in the milk of transgenic sheep (Wright et al., 1991). Similarly, the transgenic technique can also be used to improve the nutritional value of milk by selectively increasing certain valuable protein components such as caseins or by supplementing certain new and useful proteins such as lysozyme for antimicrobial activity (Maga and Murray, 1995). Second, transgenic mice have been widely used in medical research, particularly in generation of transgenic animal models for human disease studies (Lathe and Mullins, 1993). More recently, it has been proposed to use transgenic pigs as organ donor for xenotranplantation by expressing human regulators of complement

activation to prevent hyperacute rejection during organ transplantation (Cozzi and White, 1995). The development of disease resistant animals has also been tested in transgenic mice (e.g. Chen et al., 1988). Furthermore, the transgenic technique has also been widely used in plants to improve crop quality with enhanced disease resistance and preservation.

Fish are an intensive research subject for transgenic studies. The first transgenic fish report was published by Zhu et al. (1985) using a chimeric gene construct consisting of a mouse metallothionin gene promoter and a human growth hormone gene. Most of the early transgenic fish studies have concentrated on growth hormone gene transfer with an aim to generate fast growing "superfish". Because of the lack of cloned fish growth hormone gene and fish gene promoters, majority of early attempts used heterologous growth hormone genes and promoters and failed to produce gigantic superfish. However, by using "all-fish" gene constructs, i.e. fish gene promoters and fish growth hormone genes, enhanced growth of transgenic fish have been demonstrated in several fish species including Atlantic salmon (Du et al., 1992), several species of Pacific salmons (Delvin et al., 1994; 1995), and loach (Tsai et al., 1995).

Despite the success of growth hormone transgenic fish, there are still several hurdles from regulatory agencies and from psychologyical fears of cosummers because of some concerns of unsafety for food consumption and concerns of destroying the delicate ecosystem. To avoid these potential problems in future commercialization of transgenic products, we initiated a transgenic project in 1996 to introduce a fluorescent gene into fish for production of novel varieties of ornamental fish. Since this kind of transgenic fish is not food fish and is tightly controlled in aquaria, they should be readily acceptable to regulatory agencies and consumers. The basic approach we used is to insert a gene encoding GFP into the genome of the zebrafish, *Danio rerio*, under a tissue-specific promoter or a ubiquitous promoter. The selected promoter will direct the color protein to be expressed in certain tissues or ubiquitously. The GFP transgenic fish emit green light under a blue or ultra violet light. GFP has no obviously adverse effect to cellular activity and thus these fish can be used for ornamental purpose. So far, we have developed four GFP transgenic constructs: 1. pCK-EGFP, contains a skin-dominant cytokeratin gene promoter; 2) pMCK-EGFP, a muscle specific promoter from a muscle creatine kinase gene; 3) pMLC2f-EGFP, another muscle specific promoter from the myosin light chain 2 (fast muscle isoform) gene; and 4) pARP-EGFP, a ubiquitously expressed promoter from an acidic ribosomal protein P0 gene. When these chimeric gene constructs were introduced into fish, all of them showed predictable expression patterns according to the specificities of the promoters used. At present, a patent for the first three transgenic DNA constructs are being filed. This work has also attracted intensive attentions from media and our fluorescent fish has been reported in a Japanese newspaper, ***Nikkai***; and local newspapers, ***The Straight Times*** and ***Lian He Wan Bao***. It has been also reported in both Singapore television (channels 5 and 8) and radios.

In order to develop more varieties of transgenic ornamental fish, we have also partially sequenced in the past few years over 2,000 zebrafish cDNA clones and identified over 400 distinct fish cDNA clones which encode proteins for all cellular compartments and are expressed in all major tissues and organs (Gong et al., 1997; Gong, 1998). We have also developped a linker-mediated PCR method for rapid isolation of gene promoters based on partial cDNA sequence. Therefore, we are now well positioned for obtaining any types of gene promoters to target the transgene

expression in any tissue. In the present proposal, we will first use the transgenic constructs developped from the zebrafish model to other ornamental fish species including medaka, goldfish, koi carp and glass catfish. As all these species are closely related to the zebrafish, it is highly possible that all of the zebrafish gene promoters can be faithfully functional in these species. This notion is supported by the previous works using mammalian gene promoters in transgenic fish. For example, Westerfield et al. (1992) have demonstrated that two mouse hox gene promoters are correctly expressed in transgenic zebrafish; recently, Moss et al. (1996) have also demonstrated that a rat myosin light-chain enhancer can drive a reporter gene to be specifically expressed in skeletal muscles. In addition, the transgenic research has been reported for all of these species or closely related species, e.g. medaka (Ozato et al., 1986; Chong and Vielking, 1989; Gong et al., 1991), goldfish (Zhu et al., 1985; Wang et al., 1995), catfish (Hayat et al., 1991) and carp (Zhang et al., 1990).

(ii) Research experience

The principle investigator, **Dr. Gong Zhiyuan,** has been actively involved in transgenic research for the past 10 years and has hand-on experience on all techniques required in the proposed research. He has been involved in the generation of growth hormone transgenic salmon which grow over 10 times faster than wild type salmon (Du et al., 1992) and generation of cold-resistent transgenic goldfish with a fish antifreeze protein gene. In addition, He also has experience for generation of transgenic medaka for promoter analysis (Gong et al., 1991). Recently, his group in NUS has successfully generated green fluorescent transgenic zebrafish and this work is now being filed for a patent. He currently has a competent research team and his team is actively engaged in fish molecular biology research, particularly on fish gene cloning, developmental biology and transgenic fish. The co-investigator, **Professor Lam Toong Jin**, has 33 years of research experience in biological research on fish and is a prominent scientist in this field. He will be involved in marketing the fluorescent transgenic fish at late stage of the project. **Dr. Ju Bensheng** has been working on the transgenic fish project since the end of 1997. He is an expert of microinjection and fish breeding. He will take an NSTB postdoctoral fellowship in the next few months and continue to work on the project. He single-handedly set up the microinjection facility in the PI's lab and developed a variety of fluorescent transgenic zebrafish. He is pivotal to this proposed project.

III. PROGRAMME

1. Maintenance of fluorescent transgenic zebrafish

Currently, we are screening the F1 generation of GFP transgenic zebrafish and this work is expected to be completed by March 1999. For stable lines of transgenic fish, we need to continue to observe for several generations. Thus, we seek the support from the proposed grant to maintain the GFP transgenic zebrafish. Currently, we are developing three lines of GFP transgenic zebrafish: skin-specific, muscle-specific and ubiquitous. The skin-specific transgenic zebrafish express green fluorescence only from skin, the muscel-specific transgenic fish display green fluorescence from muscles, and ubiquious transgenic zebrafish express green fluoreescence from all cells.

2. Development of multi-color fluorescent transgenic zebrafish

As more fluorescent protein genes are available, it is possible to produce other color fluorescent transgenic fish. At present, in addition to GFP gene, BFP (blue

fluorescent protein), CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) genes are also available from Clontech. These new fluorescent protein genes present us the opportunity to generate multiple color fluorescent transgenic fish. With the wide range of tissue specific promoters, we will be able to generate colorful chimerical transgenic fish, for example, green skin/blue muscle/yellow eyes and many other combinations. The promoters we used successfully to drive transgenic GFP expression will be used to link these new fluorescent genes, including the skin-specific promoter from a cytokeratin gene, two muscle-specific promotors from a creatine kinase gene and a myosin light chain 2 gene, and a ubiquitous promoter from the acidic ribosomal protein P0 gene. We will develop single color transgenic fish first and then generate multi-color transgenic fish by breeding. For example, if a green muscle transgenic line crosses with a blue skin transgenic line, offsprings with green muscle and blue skin will be obtained.

3. Generation of stress-inducible GFP transgenic fish for a biosensor system

Using a stress-inducible gene promoter, a transgenic fish, where expression of transgene occurs only under certain conditions such as elevated temperature (heat shock) or exposure to a high level of heavy metals, will be produced. A suitable promoter is from the zebrafish heat shock protein 25 (hsp25) gene. This gene has been thoroughly characterized recently by an Honors student in the PI's lab (Kee, 1998), and its expression increased dramatically and rapidly upon heat-shock and also increased significantly after a long term of heavy metal (e.g. cadmium, mercury and zinc) exposure. A short promoter (about 300 bp) has been isolated for transgenic studies. In future, a longer promoter will be isolated to ensure the full response by heat-shock and heavy metals. The promoter will be linked to the GFP gene and introduced into zebrafish. The transgenic fish generated by a heavy metal inducible promoter will be useful to develop a biosensor system to monitor aquatic enviromental pollution such as by heavy metals. The expression of GFP will signal the presence of significant amount of polluants. A such biosensor system has obvious advantages over classical analytical methods because the former is rapid, visualizable, and capable of identifying specific compounds directly in complex mixture found in aquatic environment (Peter et al., 1996). Moreover, the biosensor system also provides information on biotoxicity and it is biodegradable and regenerative.

Similarly, a biosensor system to monitor hormone contamination such as by estrogen and its derivatives may be similarly developed by using an hormone inducible promoter such as from the liver-specific and estrogen inducible vitellogenin gene promoter. Currently, we have isolated five distinct zebrafish vetellogenin cDNA clones and these clones will be used for isolation of their promoters.

4. Production of fluorescent ransgenic medaka

Medaka is chosen because it is another popular fish model for genetic and transgenic studies. The transgenic techniques in this species has been well established. For example, DNA can be injected into the pronuclei of oocytes before feretilization (Ozato et al. 1986) and can also be directly injected into cytoplasm after fertilization (Chong and Vialkind, 1989). Previously, we also generated transiently expressed transgenic medaka by injection of DNA into fertilized eggs (Gong et al., 1991). We will inject all four existing zebrafish gene constructs: pCK-EGFP, pMCK-EGFP, pARP-EGFP and pMLC2f-EGFP. Transgenic expression will be monitored continuesly and stable transgenic lines will be developed a la zebrafish. Another

advantage to use medaka over zebrafish is its tissue clarity and thus it is possible to examine GFP expression in internal organs.

5. Production of fluorescent transgenic goldfish

Goldfish is a popular ornamental fish and there are hundreds of varieties. The transgenic technique has already been developed by Zhu et al. (1996) and by Wang et al. (1995) (Dr. Gong is also a co-author of the latter work). We will inject all four existing zebrafish gene constructs: pCK-EGFP, pMCK-EGFP, pARP-EGFP and pMLC2f-EGFP. Transgenic expression will be monitored continuesly and stable transgenic lines will be developed a la zebrafish. Different varieties of fluorescent goldfish can be obtained by classical breeding between fluorescent transgenic goldfish and different goldfish varieties.

6. Production of fluorescent transgenic koi carp

Koi carp is a large size ornamental fish with high commercial value. For example, a large koi of about one foot long has a value of over $1,000 in the market. Thus, fluorescent transgenic koi will have obvious commercial value. For this species, we will use the constructs we already generated from the last project, skin-specific, muscle specific and ubiquitously expressed. We will inject all four existing zebrafish gene constructs: pCK-EGFP, pMCK-EGFP, pARP-EGFP and pMLC2f-EGFP. Transgenic expression will be monitored continuesly and stable transgenic lines will be developed a la zebrafish.

7. Production of fluorescent transgenic glass catfish

The galss catfish was chosen because of its crystal clarity of the whole body and thus it is feasible to detect any tissue specific expression of GFP. Some organ-specific promoters, such as liver-specific, intestine-specific, bone-specific, brain-specifc and heart specific promoters, can be used for this species. Therefore, we will isolated these organ-specific promoters for construction of GFP chimeric genes and thus more varieties of fluorescent ornamental fish can be produced from this species. At the begining, We will inject all four existing zebrafish gene constructs: pCK-EGFP, pMCK-EGFP, pARP-EGFP and pMLC2f-EGFP. Transgenic expression will be monitored continuesly and stable transgenic lines will be developed a la zebrafish.

IV. RESOURCE

(i) Manpower Cost

This project is a continuation of the previous transgenic ornamental fish project (RP960315), which has a manpower support for a Lab Technologist, **Dr. Ju Bensheng**. Dr. Ju will be offered an NSTB postdoctoral fellow for two years starting in the next few months and will continue to work on the proposed project. Therefore, we propose a Research Assistant (Hons) only for 36 months starting from the second half year of the project (around February 2001). The research assistant will continue Dr. Ju's work and an overlap of six months with Dr. Ju will be important to ensure a smooth transition of the project. Currently the PI has a research assistant, Ms. He Jiangyan, under the neurogenic pathway grant (RP3972393) and her appointment will be ended by February 2001 when the grant is terminated. In addition, one part-time undergraduate student assistant will be recruited each year for the proposed project and they will work four month each year at a salary scale of $500 per month.

(ii) New Equipment/Facilities costs

Majority of the laboratory equipment has been provided by my previous grants and will be used by the new project. In the present grant, we only request a -20°C freezer. Currently we have only one such freezer which is being shared by 10 long-term researchers (excluding Honors students and occasional project students), and in the past few years, tremendous amount of biological samples has been generated by our highly active research activity and need to be stored at -20°C. We anticipate more samples will be generated when the new project is to start. Thus a new freezer is required to accommodate the biological samples.

(iii) Materials and Consumables:

The items of consumables are listed in section 8.3.

(iv) Miscellaneous costs

These include taxi fare for transportation of live fish ($600) and miscellaneous costs including IDD calls, FAX, courier service, photocopy, stationary, patent search etc. ($3,000).

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Collaborator: Lam Toong Jin

Academic qualifications: 1965 - BSc Hons Zoology (First Class)
Univ of British Columbia, Canada

1969 - PhD (Univ of British Columbia)

Awards/Honours:

Colombo Plan Scholarship, 1962-1969
CIDA-NRC Research Associateship (Canada), 1974-1976 (3 months each yr)
National Science & Technology Award (Singapore), 1990 (for outstanding contributions to Science)
Fellowship of the Zoological Society, Calcutta (F.Z.S., Cal.) Honors Causa, 1991
10th W.S. Hoar Lecturer (1997), Department of Zoology, University of British Columbia

Present Position: Professor & Head
Department of Biological Sciences, NUS

Research experience: 32 years of research in fish physiology

Publications:

Over 150 publications (excluding conference abstracts)

Curriculum Vitae: Dr. Zhiyuan Gong

Education:
BSc (1982) Ocean University of Qingdao, China
Ph.D (1987) McGill University, Canada

Work Experience:
1987-1988: Postdoctor, McGill University, Canada
1988-1995: Research Fellow, Hospital for Sick Children, Toronto, and University of Toronto, Canada
1995-1997: Lecturer , National University of Singapore, Singapore
1997-present: Senior Lecturer, National University of Singapore, Singapore

Research Grants:

1. RP950304 ($209,435, 07/95-03/98): Developmental regulation and functional analysis of a family of LIM domain homeobox genes in zebrafish.
2. RP954346 ($70,300, 07/95-07/97): Identification of surface antigens in *Ichthyophthirius multifiliis* and the development of fish vaccine.
3. RP960315 (S$114,500, 09/96-09/99): Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP).
4. RP3972393 ($249,457, 08/97-08/2000): Molecular dissection of neurogenic pathway in zebrafish.

Publications:
Number of papers in internationsl journals: 37;
Number of invited reviews and book chapters: 6;
Number of conference papers: 65.

10 Relevant Publications:

1. Gong, Z., C.L. Hew, and J.R. Vielkind (1991) Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese Medaka embryos. *Mol. Marine Biol. Biotech.* 1: 64-72.
2. Du, S.J., Z. Gong, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler, and C.L. Hew (1992) Growth enhancement in transgenic Atlantic salmon by use of fish antifreeze/growth hormone chimeric gene constructs. *Bio/Technology* 10:176-181.
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Personal

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Employment

July 1992- May 1994	Assistant lecturer in Fisheries College, Ocean University of Qingdao (OUQ), P.R.CHINA
Dec. 1997-	Lab. Technologist in School of Biological Sciences, National University of Singapore

Education

Dec. 1998	Obtained my Ph.D. degree from Department of Biological Sciences, National University of Singapore
July 1992	Obtained my M. Sc. degree from Fisheries College, OUQ
July 1989	Obtained my Bachelor's degree from Fisheries College, OUQ

Publications

1. B., Ju (1992) Inducing triploidy in the Red Sea Bream *Pagrus major*. Proceedings of the Third Asian Fisheries Forum, Singapore, p91- 97.
2. B., Ju and H. W., Khoo (1997) Transient expression of two luciferase reporter gene constructs in developing embryos of *Macrobrachium lanchesteri* (de Man). Aquaculture Research, 28, 183-190.
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4. B., Ju and H. W., Khoo Characterisation of Crustacean Hyperglycemic Hormone cDNAs and genomic structures in the shrimp *Macrobrachium lanchesteri* (de Man) (Submitted to GENE)